ISOLATION, PURIFICATION AND CHARACTERIZATION OF
AN EXTRACELLULAR PROTEOLYTIC ENZYME OF Planococcus citreus

BY

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A mis queridos padres:

    Gracias por la ayuda brindada, el amor,
el apoyo moral y la vision de avanzar en la vida.

                        Con todo mi amor

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Planococcus citreus is a gram-positive marine bacterium commonly
found in fresh and iced shrimp. Various studies have indicated that it
may contribute to spoilage of this valuable marine resource. In order
to understand the contribution of this organism to the degradation of
shrimp as well as other proteins, an investigation was undertaken to
study the extracellular proteolytic enzyme(s) of this organism. Results
indicated that the major portion (>95.0%) of the proteolytic activity
resided in the extracellular fraction.

Under the conditions tested, maximum extracellular enzyme produc-
tion occurred in Trypticase Soy Broth (TSB) as observed by the highest
m value (units of enzyme activity per cell per hour). In addition, the
cell-free broth obtained from P. citreus cells grown at 5 C for 108 hrs,
20 C for 72 hrs and 35 C for 36 hrs exhibited enzyme activity towards
shrimp protein at all three enzyme-substrate incubation temperatures (5,
20 and 35 C).

P. citreus was grown in Trypticase Soy Broth at 20 C for 72 hrs. Centrifugation, ammonium sulfate precipitation, Sephacryl[R] S-200 Super-fine molecular sieve chromatography, DEAE-Sephadex[R] A-50 ion exchange chromatography and acrylamide gel electrophoresis were used to purify the extracellular enzyme(s). The enzyme was purified 26.50 fold (using the fluorometric technique for activity measurement), and recovery of the enzyme was above 49%. Gelatin and shrimp protein were used as sub-strates throughout the study. The molecular weight of the purified protease was approximately 29,000 as measured by Sephacryl[R] S-200 column chromatography and acrylamide gel electrophoresis.

Maximum activity of the enzyme was at pH 8 and 35 C. Ionic strengths of above 0.83 (0.75 M NaCl) decreased the activity of the extracellular enzyme. Heat treatment at 65 C for 15 min destroyed the activity of the purified enzyme. However, 1.0% of the residual enzyme activity was still present in the cell-free broth of _P. citreus_ grown at 35 C for 36 hrs. In contrast, 15 min at 75 C were necessary to reduce 99.0% the activity of the enzymes in the cell-free broth of _P. citreus_ grown at 5 C for 108 hrs. When shrimp protein was used as substrate, sodium chloride concentrations of 0.0-0.5% increased enzyme activity, while concentrations of 0.5-1.5% decreased enzyme activity. However, when gelatin substrate was used, NaCl concentrations of 0.0-1.5% had no effect on enzyme activity. The activity of the purified enzyme decreased as the concentration of sodium bisulfite increased. Michaelis-Menten kinetics were followed when gelatin and shrimp protein preparation were used as substrates. The apparent K_m values for gelatin and shrimp protein were 0.98 mg/ml and 0.33 mg/ml, respectively. The apparent V_{max} values were 666.67 and 431.03 units of activity for gelatin

and shrimp protein, respectively. Ferric chloride, mercuric chloride, potassium chloride, ethylene diaminetetraacetic acid, citric acid, cysteine, p—mercaptoethanol, potassium permanganate and formaldehyde partially inactivated the enzyme. Calcium chloride increased the activity of the extracellular proteolytic enzyme. Zinc chloride, p—dioxane, manganese chloride and magnesium chloride had no effect on the activity of the enzyme. The proteolytic enzyme exhibited peptidase activity on various commercial synthetic dipeptides. The extracellular proteolytic enzyme produced by _P. citreus_ was apparently not induced by the presence of shrimp protein in the medium of growth. Enzyme production appeared to be related to the extent of growth of _P. citreus_ in the medium.

INTRODUCTION

Quality deterioration and subsequent spoilage of shrimp during storage are caused primarily by activities of indigenous tissue enzymes and microbial enzymes (69). Various researchers (17,156) believe that bacterial action plays a more important role than autolytic enzyme release in causing spoilage of seafoods. During growth of the bacteria, proteolysis of shrimp proteins and free amino acid formation by microbial action has been observed. Enzymatic deamination and decarboxylation of these amino acids from shrimp protein occur, resulting in the formation of malodorous compounds (116).

Various types of bacteria have been reported to be present on freshly caught shrimp. Numerous studies (3,42,43,45,116,142) have shown the changes undergone by the bacterial flora of shrimp as the storage period increases. Recent research (1,2,3,90) has noted the presence of a gram-positive organism, Planococcus citreus, during shrimp storage. The organism is described as a motile gram-positive coccus found in the marine environment, capable of growing over a pH range of 7-10 between 5-35 C in broth containing 0.5-12% sodium chloride (NaCl), and the organism is capable of hydrolyzing gelatin, cottonseed, soy and shrimp protein.

The potential of P. citreus as a "spoiler" of shrimp was shown by the increase in pH and the rapid increase in the total volatile nitrogen/amino acid-nitrogen ratio (TVN/AA-N) and trimethyl-amine nitrogen (TMN) following growth of this organism on shrimp (4). The proteolytic

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activity of this organism was further demonstrated by the decrease in percent total extractable protein (percent TEP) in shrimp during storage at 5 C (4,5) which had been inoculated with P. citreus.

The proteolytic activity exhibited by this organism deserves additional research in order to better understand the contribution of P. citreus to the degradation of shrimp protein. A study was therefore undertaken to study the enzyme(s) responsible for protein degradation. The optimum medium and stage in the growth cycle of P. citreus were determined for maximum extracellular enzyme(s) production. The effect of incubation temperature (5, 20 and 35 C) on the growth of P. citreus and proteolytic enzyme production was also investigated. Purification of the extracellular enzyme(s) was achieved by precipitation and chromatographic techniques. Homogeneity of the enzyme was evaluated by gel electrophoresis and chromatographic techniques. Optimum pH and temperature, ionic strength effect, thermal stability, molecular weight, sodium chloride effect, sodium bisulfite effect, enzyme concentration, substrate concentration, and the effect of metal ions and other reagents were investigated. In addition, the potential of the P. citreus enzyme(s) to degrade dipeptides and the possible effect of shrimp protein in the growth medium inducing the extracellular enzyme(s) were studied.

Results obtained from this investigation indicate that P. citreus, while growing on shrimp, may contribute to the overall decrease in shrimp quality during iced or refrigerated storage. In addition, information about the characteristics of the enzyme(s) produced by P. citreus will be introduced.

LITERATURE REVIEW

In 1978, the shrimping industry was the most valuable fishery in the United States (8). However, the quality of shrimp often falls short of that expected by the consumer. Approximately 15-20% of the shrimp landed is eventually lost due to quality deterioration. This deterioration of shrimp quality is usually attributed to rapid bacterial enzymatic changes of the fresh shrimp resulting from mishandling and/or inadequate processing. These changes, along with the chemical and physical methods for measuring shrimp quality, are discussed as a basis for the investigations presented in this dissertation.

Shrimp Spoilage

Quality changes in shrimp during storage on ice can lead to major economic losses in the shrimp industry. Mechanical damage, bacterial contamination and enzymatic activity may combine to cause undesirable changes in the composition and quality of shrimp (20,34,38,39,43,45,53, 54,77,78,79,89,94,108,117,153,154).

The loss of acceptability of shrimp may be triggered by several factors: 1) shrimp muscle enzymes, 2) direct microbial activity, 3) microbial enzymes and/or 4) a combination of these factors. Defects which may occur as a result of such reactions are formation of malodorous substances, flavor deterioration, toughness, mushiness, juiciness, dryness and discoloration (116).

Proteolytic enzymes play an important role in the spoilage of shrimp by degrading muscle proteins and polypeptides, forming amino acids which

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enrich the natural substrates and are thus available for the growth of microorganisms. Enzymatic deamination and decarboxylation of amino acids may also occur rapidly, resulting in the formation of spoilage products. Pedraja (116) observed that from the moment a shrimp is taken out of the water, its free amino acid pool is affected to some extent by osmoregulation and also by the struggle during catching. Therefore, the onset of enzymatic and bacterial actions will vary according to the factors affecting the substrates available in shrimp muscle.

Another factor that can induce shrimp spoilage is mechanical damage. Handling shrimp on the boats results in mechanical damage to the muscle, which will accelerate microbial invasion. The expressible fluid with its protein and amino acid content serves as an excellent medium for growth and reproduction of invading microorganisms (116).

Microbiological Characteristics of Shrimp

The muscle tissue of freshly caught shrimp is generally regarded as sterile (26); however, work by Lightner (97) showed bacteria in the gut, gills and between muscle bundles of brown shrimp. Reports on the number of bacteria found on freshly caught shrimp range from 2.5×10^2 to 2.0×10^6 organisms per gram (org/g) with Gulf coast shrimp averaging 1.0×10^4 org/g, whereas bay shrimp averaged 1.0×10^5 org/g (31,34,39,43,78,142). Work completed in our laboratory has shown that fresh shrimp from the Gulf of Mexico had bacterial counts ranging from 4.0×10^5 org/g to 2.0×10^6 org/g, while shrimp from the Atlantic coast had bacterial counts ranging from 4.5×10^5 to 3.6×10^6 org/g (1).

Various kinds of bacteria have been reported on freshly caught shrimp. Initially, the microbial flora is a mixture of organisms from both the marine and terrestial environment. In the early 1950s, Campbell

and Williams (31) and Williams et al. (154) isolated species of <u>Achromo-</u>
<u>bacter</u>, <u>Bacillus</u>, <u>Micrococcus</u>, <u>Flavobacterium</u> and <u>Pseudomonas</u> from Gulf
coast shrimp. Vanderzant et al. (142) reported that the flora of shrimp
from the Gulf of Mexico consisted of coryneforms, <u>Achromobacter</u>, <u>Flavo-</u>
<u>bacterium</u> and <u>Bacillus</u>. In Pacific shrimp, <u>Acinetobacter-Moraxella</u> spe-
cies were predominant (80). Lee and Pfeifer (94) reported that the flora
of Pacific shrimp (<u>Pandalus jordani</u>) consisted of <u>Moraxella</u>, <u>Pseudomonas</u>,
<u>Acinetobacter</u>, <u>Arthrobacter</u> and <u>Flavobacterium-Cytophaga</u> species. Cann
(32) and Cann et al. (33) found that coryneform organisms were predomi-
nant in the bacterial flora of scampi, <u>Nephrops norvegicus</u>, with strains
of <u>Achromobacter-Acinetobacter</u> group and <u>Pseudomonas</u>, <u>Cytophaga</u> and
<u>Micrococcus</u> species also present. Koburger et al. (90) reported that
the <u>Flavobacterium-Cytophaga</u> group represented the majority of the
organisms of fresh rock shrimp (<u>Sicyonia brevirostris</u>), and Alvarez (1)
and Alvarez and Koburger (3) reported that <u>Flavobacterium</u> and <u>Pseudomonas</u>
were the predominant groups isolated from <u>Penaeus</u> shrimp from the East
and West coasts of Florida.

When shrimp are stored in ice, the number and kinds of bacteria
shift to a predominantly psychrotrophic flora (130). Psychrotrophs are
described as organisms having an optimal growth temperature of about 20
C. A comparatively longer storage life of iced shrimp from tropical
waters has been reported by Carrol et al. (34). Cann et al. (33) in
their review on tropical shrimp indicated that penaeid shrimp from the
Gulf of Thailand remained in acceptable condition for 12-16 days on ice,
whereas nontropical shrimp, such as <u>Pandalus</u> and <u>Nephrops</u> species, were
totally spoiled after 8-10 days. They attributed this difference to the
bacterial flora; the mesophilic flora on tropical shrimp are not active

at ice temperatures and little spoilage occurs until the psychrotrophic
flora develops. Cann et al. (33) stated that the amount of spoilage may
be related to the degree to which psychrotrophic strains are introduced
with the ice. Consequently, the rate of increase in bacterial growth
depends on the initial number of bacteria, handling on deck, and amount
and quality of ice used. Shewan (130) demonstrated that the action of
many psychrotrophic organisms resulted in rapid fish spoilage. The
principal organisms he mentioned were Pseudomonas, Aeromonas, Vibrio,
Moraxella, coryneforms and Flavobacterium. Castell and Mappleback (35)
concluded that Flavobacterium was among the most important of the fish-
spoilage bacteria. Flavobacterium is a frequently encountered bacterium
on fresh shrimp flesh.

The bacterial flora of shrimp undergoes marked changes as the stor-
age period increases. Campbell and Williams (31) showed Bacillus, Micro-
coccus and Flavobacterium made up over 50% of the flora initially,
whereas the Achromobacter-Pseudomonas group accounted for 98% of the
flora after 16 days of iced storage. In a study on the bacterial spoil-
age patterns of headless brown shrimp, Cook (45) noted that there was
only one consistent change in the bacterial types growing initially or
during the period of die-off. As the bacterial count began to rise,
Pseudomonas species became the predominant organism, accounting for 80-
100% of the bacterial types isolated. Vanderzant et al. (142) reported
that the predominant bacterial flora of fresh shrimp consisted of
coryneforms and that following storage Pseudomonas species predominated.
Cobb et al. (43) indicated that typical spoilage organisms of the genus
Pseudomonas are not usually found in freshly caught shrimp. It is not
until the shrimp are exposed to handling on board the vessel that this
organism becomes apparent.

Alvarez and Koburger (3) reported that the numbers of <u>Moraxella</u>, <u>Vibrio</u>/Aeromonas and <u>Planococcus</u> species isolated from <u>Penaeus</u> shrimp remained relatively constant throughout 10 days of ice storage. However, <u>Flavobacterium</u> isolates increased until the fifth day, then decreased rapidly. <u>Pseudomonas</u> species showed the opposite trend. They decreased until the fifth day, then increased rapidly. Other workers have observed the presence of <u>Flavobacterium</u> in raw shrimp (31,80,90,94,142,143) and have noted this decrease in numbers during ice storage with a subsequent increase in <u>Pseudomonas</u> species. Cook (45) was unable to produce typical spoilage when shrimp were inoculated with <u>Flavobacterium</u> species, indicating that they are probably an inert group of organisms found in shrimp. In contrast, <u>Pseudomonas</u> species have been implicated as the organisms primarily responsible for the spoilage of marine products stored in ice (108,130).

Measurement of Shrimp Spoilage

Numerous methods for determining shrimp quality have been developed; however, due to the complexity, time involved and inconsistent results of many of these methods, only a few are routinely used by the industry and then, only for internal quality control. In many of these chemical tests, results can vary with the age of the shrimp, size, species, area of catch and handling conditions. Many of the tests only indicate the onset of spoilage (31,109). Table 1 lists the chemical and physical tests that have been used to measure shrimp quality. Total volatile nitrogen/amino acid-nitrogen (TVN/AA-N) ratio (40,41,42,43,64,75) is the chemical test that shows the best correlation with organoleptic quality measurements of shrimp. Moore and Eitenmiller (107) compared various methods for measuring shrimp quality. They observed that a relatively

Table 1. Chemical and physical tests available to measure shrimp quality.

Test	Parameter Measured	Reference
acid-soluble orthophosphate	trichloroacetic acid and soluble orthophosphate	14
adenosine triphosphate and its degradation products to hypoxanthine	degradation of adenine dinucleotides to hypoxanthine	64
alcoholic tumeric solution	changes in percent transmission of a yellow tumeric shrimp solution	75
amino-nitrogen	changes in amine nitrogen content	14,19,62,93
ammonia	ammonia content	75,143
B-vitamin content	content of B-complex vitamins	15
cathecol ferric chloride	change in percent transmission of shrimp filtrate mixed with cathecol ferric chloride	85
dimethylamine	degradation of trimethyl amine oxide by enzyme (TMO) to DMA	31
direct microscopic counts	actively and non-actively metabolizing bacteria	109
fluorescamine	changes in free amine fractions	107
free fatty acids	percent of free faty acids	31
glycogen	glycogen content	14,93

Table 1. (continued)

Test	Parameter Measured	Reference
hydrogen sulfide	measure of H_2S presence	31
hydration capacity	hydration of water insoluble protein	14,129,141
inosine monophosphate	degradation of adenine dinucleotides to IMP	132
indole	utilization of tryptophane by bacteria and its conversion to indole	31,64,75,93
iodine titration	presence of iodine	14,64,75
lactic acid	lactic acid content	14
methylene blue reductase	reduction of methylene blue by bacteria	111
peroxide number	determines peroxide oxygen which has formed at the double bonds in unsaturated fatty acids	31
pH	hydrogen ion concentration	14,19,68,69,85, 93,141
phenol red test paper	changes in pH	86
photoelectric reflection number	changes in light transmission of shrimp extract	64
picric acid	turbidity of shrimp filtrates with picric acid	19
skatole	production of skatole	93

Table 1. (continued)

Test	Parameter Measured	Reference
total fat	fat content	93
total nitrogen	ammonia content	93
trimethylamine nitrogen	degradation of trimethylamine oxide by enzyme (TMO) to TMA	14,19,31,44,64,67,75,85
total volatile nitrogen	volatile nitrogen compounds (ammonia)	64,75
tyrosine	free tyrosine levels	67
ultraviolet light-change in fluorescence	ultraviolet absorption of shrimp extracts	93
volatile acids	volatile acidic compounds	19,31,46,62,68,69
volatile reducing substances	measure of volatile nitrogen compounds	62
volatile nitrogen	volatile nitrogen containing compounds (ammonia)	85

new method using fluorescamine primarily detected only the non-protein,

non-ammonia, small molecular weight amines in shrimp homogenates. They

proposed that fluorescamine analysis could be useful in determining

changes in the free amine fractions. The shrimp industry still depends

on visual observation, smell and bacteriological testing for evaluating

overall shrimp quality, whereas, the Food and Drug Administration (FDA)

uses decomposition, filth and odor for the evaluation of shrimp quality.

Characteristics of _Planococcus citreus_

Koburger et al. (90) noted the presence of a high percentage of

gram-positive organisms following iced storage of rock shrimp (_Sicyonia_

brevirostris). These organisms comprised up to 68% of the isolates. Of

these 40% were _Planococcus citreus_, an aerobic gram-positive motile

coccus of marine origin producing an orange or yellow pigment. Informa-

tion describing the isolation and characteristics of this organism is

limited. _P. citreus_ was previously named _Micrococcus citreus_ (27). The

8th Edition of the _Bergey's Manual of Determinative Bacteriology_ (29)

does have a description of the organism; however, it is limited in scope.

Cook in 1970 (45) and previous researchers working with shrimp placed

all aerobic gram-positive to gram-variable coccoid shaped bacteria in

the genus _Micrococcus_. In addition, Cook (45) noted that many of these

organisms isolated from shrimp were pigmented orange or yellow and were

motile. According to Bergey's manual (29), the only genus in the family

Micrococcaceae that is pigmented, either yellow or orange, and motile,

is _Planococcus_. This change in the taxonomic status of this organism

and the difficulty of demonstrating motility are probably the reasons

why _Planococcus_ has not been reported in previous studies.

The taxonomic status of _Planococcus citreus_ has changed markedly

through the years. In 1894 and again in 1900, Migula (103,104) made a

recommendation that flagellated cocci be included either in the genus
Planococcus or Planosarcina. This suggestion was accepted by only a few
authors, e.g., Krasil'nikov in 1949 (92). The majority of the authors
have included the flagellated cocci in the genus Micrococcus (22,83),
mainly because these cocci could only be differentiated from the other
members of the genus by their motility. Most authors have considered
motility to be a minor characteristic for the recognition of a new genus.
The findings of Bohacek et al. (23,24) that the flagellated cocci differ
considerably in the guanosine-cytosine (GC) content of their deoxyribo-
nucleic acid (DNA) from other cocci shed new light on their taxonomic
position. It was proposed by Bohacek et al. (23) to include the flagel-
lated cocci with a GC content ranging from 40-50% in the genus Plano-
coccus. In 1970, Kocur et al. (91) revised and outlined the genus
Planococcus. However, according to Index Bergeyana (86), the Planococcus
genus includes nine species (P. agilis, P. casei, P. citreus, P. citro-
agilis, P. europeans, P. loffleii, P. luteus, P. ochrolencus and P.
roseus). Kocur et al. (91) evaluated the strains available in culture
and proposed that seven belong to one species, Planococcus citreus.
Although the remaining two species were closely related, he refrained
from giving a precise designation and labeled them only as Planococcus
species.

Schleifer and Kandler (126) found that the strains studied by
Bohacek et al. (23,24) and Kocur et al. (91) were uniform with respect
to the type of murein present in their cell walls and similar to that
of members of the genera Micrococcus and Staphylococcus. However,
serological investigation of P. citreus by Oeding in 1971 (112) revealed
no antigenic relationship to staphylococci or micrococci.

Thirkell and Summerfield (137,138) studied the effect of varying the sea salt concentration on the chemical composition of a purified membrane fraction of P. citreus. They concluded that the concentration of salt in the medium affected the amount of membrane in the cell. Salt concentrations above or below the normal 3% of sea water reduced the amount of membrane material present. In addition, varying salt concentration had no significant effect on the amount of total neutral lipid, glycolipid or phospholipid in the P. citreus membrane preparations. But a significant effect was observed on the amount of individual neutral lipid or phospholipid classes present and on the number of individual glycolipid components detected.

Our attention was directed toward this organism when, during a study of the normal flora of rock shrimp (Sicyonia brevirostris), P. citreus was consistently isolated and found to increase in numbers during iced storage (90). In this study, 68% of the isolates recovered were gram-positive cocci, with P. citreus increasing from 10% of the isolates on the fresh rock shrimp to 40% on the ice stored rock shrimp. In recent work (1,2,3), P. citreus has been found to be an important member of the normal flora of Penaeus shrimp.

Alvarez and Koburger (5) described P. citreus as a motile gram-positive coccus found in the marine environment, capable of growing over a range of pH 7-10, 5-35 C, in broth containing 0.5-12% sodium chloride (NaCl) and capable of hydrolyzing gelatin, cottonseed, soy and more importantly to seafood microbiologists, shrimp protein. Figure 1 shows a photomicrograph of P. citreus illustrating its morphology and flagellation. Table 2 shows the capabilities of this organism to hydrolyze various protein sources.
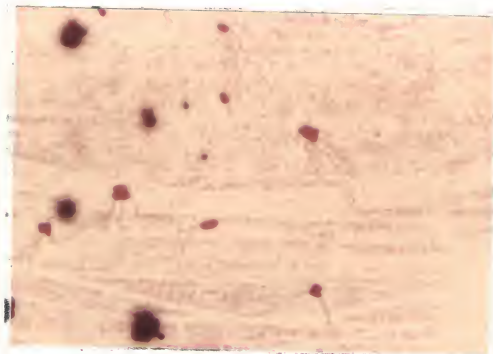
Figure 1. Photomicrograph of <u>Planococcus</u> <u>citreus</u> cells showing morphology and flagellation (6) (magnification 950X).

Table 2. Hydrolysis of various protein sources by selected strains of _Planococcus citreus_ at 25 C (4) (modification of Frazier (72)).

Isolate	Gelatin[1]	Whey[2]	Cottonseed[2]	Casein[1]	Shrimp[3]	Fish[3]	Peanut[2]	Corn germ[2]	Soy[2]	Hog blood isolate[4]	Barley[2]
A 17	+[5]	+	+	−	+	−	−	−	+	+	−
E 4	+	+	+	−	+	−	−	−	+	−	−
E 1	+	+	+	−	+	−	−	−	+	+	−
E 7	+	+	+	−	+	−	−	−	+	+	−
F 9	+	−	+	−	+	−	−	−	+	+	−
F 15	+	−	+	−	+	−	−	−	+	−	−
F 18	+	−	+	−	+	−	−	−	+	−	−
KS-1	+	−	+	−	+	−	−	−	+	−	−
KS-2	+	+	+	−	+	−	−	−	+	−	−
KS-3	+	−	+	−	+	−	−	−	+	−	−
KS-4	+	+	+	−	+	−	−	−	+	−	−
CS-1	+	+	+	−	+	−	−	−	+	−	−

[1]From Difco Laboratories, Detroit, MI.

[2]Protein isolates obtained from Southern Utilization Research and Development Division, New Orleans, LA.

[3]Fresh samples were diluted 1:10 with 0.05 M phosphate buffer pH 7 and ground in a Waring blender, dialyzed overnight with 10 volumes of the same buffer (5 C) and lyophilized.

[4]F. W. Knapp, Food Science and Human Nutrition Department, University of Florida, Gainesville, FL.

[5]+ = hydrolysis; − = no hydrolysis.

The reports (4,5) by Alvarez and Koburger outline some observations on the distribution of P. citreus in the marine environment. Of the 35 samples of marine origin examined for P. citreus, only 5 yielded this organism. Four were shrimp samples and the fifth was a stuffed flounder sample that had been prepared in a plant that processed predominantly shrimp. One of the shrimp samples from which Planococcus was isolated had been in frozen storage for over six years. Fresh seafood (trout, sheephead, mackerel, crab and oysters) as well as Gulf Coast waters and sediments from the vicinity of Suwannee, Florida, were also examined for P. citreus without success. However, in more recent studies performed by Mallory et al. (100), P. citreus was isolated from estuarine areas of Chesapeake Bay in low numbers.

Since the isolation of gram-positive organisms from iced seafood is uncommon, Alvarez and Koburger (5) studied the contribution of P. citreus to the spoilage of Penaeus shrimp. They utilized gamma irradiation (600 Krads) to lower the number of bacteria in raw shrimp and then inoculated a portion of the shrimp with 5×10^3 P. citreus cells per gram of shrimp in order to study the changes produced by this organism. P. citreus counts increased in the inoculated shrimp from 5×10^3 bacteria/gram at 0 day to 1.9×10^8 bacteria/gram at the 16th day. The potential of P. citreus as a "spoiler" of shrimp was shown by an increase in pH and the rapid increase in total volatile nitrogen/amino acid-nitrogen ratio (TVN/AA-N) and trimethyl-amine nitrogen (TMN) content. In 1973, Cobb et al. (42) reported a high correlation between total volatile nitrogen/amino acid-nitrogen ratio (TVN/AA-N) and quality of shrimp. Later work (41) suggested that the TVN/AA-N ratio and the logarithm of bacterial counts increased at approximately the same rate after the initial lag phase of

bacterial growth and that a TVN/AA-N ratio of 1.3 indicated a limited
shelf life of the shrimp. Alvarez and Koburger (4,5) showed that P.
citreus is capable of increasing the TVN/AA-N ratio at a similar rate as
the control sample (natural flora of shrimp). Thus, if the TVN/AA-N
ratio is an index of shrimp quality, P. citreus is capable of shortening
the shelf life of shrimp. The proteolytic activity of this organism was
demonstrated by a significant decrease in percent extractable protein
(% TEP) in the early days of storage. Maximal percent TEP decrease was
observed between the 4th and the 12th day of storage of shrimp at 5 C
(5).

Proteolytic Enzymes

Enzymes are proteins with highly specific catalytic activities. As
catalysts, enzymes have the following properties: 1) they are effective
in small concentrations; 2) they remain unchanged in the reaction; 3) if
present in small concentrations relative to the substrate, they speed
attainment of equilibrium as reflected by increases in the rate constants
K_1 and K_{-1} (A + B $\underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}}$ C + D, where A + B = reacting substances, C + D
= products of the A + B enzyme catalyzed reaction, K_1 = rate constant of
the forward reaction, K_{-1} = rate constant of the reverse reaction).
However, an enzyme does not change the ratio $K_1/K_{-1} = K_{eq}$ (95).

Most living organisms possess the ability to degrade proteins to
more readily absorbed substances. Such attacks on the peptide bond are
made possible by the presence of proteolytic enzymes. Although proteo-
lytic enzymes from animal sources have been studied for more than a
century by both physiologists and biochemists, it was the work of Berg-
mann and Fruton (18) which led to a more complete understanding of the
mode of action of these enzymes. Their work established conclusively

that these enzymes exert a specificity toward the amino acids involved in the peptide bonds which they attack. Bergmann and his students are also responsible for the presently accepted classification of proteolytic enzymes: They proposed that these enzymes be grouped into two classes—endopeptidases and exopeptidases—depending upon whether they hydrolyzed peptide bonds remote from, or near to, the end of the peptide chains of their natural substrates. The former class includes such enzymes as pepsin, trypsin and chymotrypsin, while the latter class contains the dipeptidases and the amino and carboxy peptidases.

Proteinases in bacteria may be either intracellular or extracellular depending upon whether they exert their activity within the cell or whether they are excreted from the cell to attack proteins in the environment (10,58). Also, enzymes may be classified according to their location in, on or around the cell: a) cell-bound: 1) truly intracellular, 2) surface-bound; and b) extracellular (58). Extracellular enzymes are those enzymes which exist in the medium around the cell, having originated from the cell without any alteration to cell structure greater than that compatible with the cell's normal processes of growth and reproduction. This distinction is not always clear and in some instances it is entirely possible that autolysis of cells has permitted the escape of intracellular enzymes into the culture filtrate. This is particularly true when high proteolytic activity is dependent upon prolonged incubation of the culture (74).

In 1964, the International Union of Biochemistry (54) recommended a scheme for numbering enzymes, which is currently used for the classification of enzymes. Enzymes are divided into groups on the basis of the type of reaction catalyzed, and this, together with the name(s) of the

substrate(s), provides a basis for naming individual enzymes. Each enzyme number contains four elements; the first element (1 through 6) shows to which of the 6 main groups of enzymes the particular enzyme belongs (the six main groups are made on the basis of the general chemical reaction catalyzed); the second and third elements show the subclass and sub-subclass, respectively, thus defining the type of reaction; and the fourth element is the serial number of the enzyme within its sub-subclass. Enzymes can be divided into six main groups: oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases.

Active extracellular proteinases are produced by numerous species of Clostridium, Proteus, Bacillus, Pseudomonas, Micrococcus, Streptococcus, Escherichia, Cytophaga and Staphylococcus (11,12,36,58,59,65,70,84,87, 105,110,113,114,118,136,139,155,157).

The continued study of these bacterial enzymes is important for at least two reasons: (a) proteolysis by microorganisms plays an important role in the biogeochemical cycles (74) and is responsible for numerous environmental interrelationships; (b) the purification and the elucidation of their bond specificities are certain to lead to the discovery of new enzymes with new properties not previously known.

Measuring Proteolytic Activity

Many methods are available for measuring proteolytic activity. Some are based on the measurement of increase in protein (or nitrogen) solubility in the supernatant after centrifugation of the reaction mixture. The most frequently cited method for measuring protein in solution is that of Lowry et al. (98) in which the tyrosine-tryptophan groups of proteins in solution, or precipitated with acid, are reacted with alkaline Folin-phenol reagent after an alkaline copper treatment (71) to

produce a blue color that is measured in a spectrophotometer. Other methods record proteolysis as the increase in ultraviolet absorption at 280 nm or the increase in absorbance (660 nm) of the tyrosine-tryptophan filtrate after trichloroacetic acid (TCA) precipitation of the undigested protein reacted with diluted (2:1) phenol reagent solution (9).

Schwabe (127) described a method which permitted the assay of the proteolytic enzyme activity on hemoglobin utilizing the fluorescamine technique. The assay is about 100 times more sensitive than the Lowry method, much faster and less complicated. He observed that the two main obstacles for the successful use of fluorescamine in his assay system were (1) the high blank produced by the reaction of ε-amino groups of the protein and (2) the fluorescent quenching effect of the hemoglobin. The high blank of the hemoglobin he substantially suppressed by a chemical modification, i.e., succinylation. Hemoglobin is usually used as a 2% solution of which only 10 μl are pipetted into 2 ml of phosphate buffer used for the reaction. He observed that the enzyme activity as measured by the fluorescamine method remained linear throughout thirty minutes while the Lowry method indicated a definite slowing of the reaction beginning at about ten minutes. This was due to the fact that fluorescamine detects an increase in free amino groups while the Lowry reagent as well as the direct measurement of absorption at 280 nm depends on the production of tyrosine or tryptophan containing peptides. A possible explanation for this discrepancy is that the enzyme in its initial attack on the hemoglobin molecule releases large peptides which are TCA soluble and that subsequent enzyme action further degrades these large peptides without significantly increasing the number of TCA-soluble fragments containing tyrosine or tryptophan moieties. A reagent depending upon primary amine groups is not subject to this error (125).

Fluorescamine is a new reagent for the detection of amino acids, peptides, proteins and primary amines in the picomole range (18,133,140). Its reaction with amines is almost instantaneous at room temperature in aqueous media. The products are highly fluorescent, whereas the reagent and its degradation products are nonfluorescent.

McCaman and Robins (101) introduced a fluorometric method now widely used for assay of serum phenylalanine which is based on the interaction of ninhydrin and peptides. Samejima et al. (124,125) found that it was the phenylacetaldehyde formed on interaction with ninhydrin which combined with additional ninhydrin and peptide or any other primary amine to yield highly fluorescent products. The structure of these products was subsequently elucidated by Weigele et al. (145), who then synthesized a novel reagent (145). This reagent 4-phenylspiro (furan-2(3H),I'-phthalan) 3,3'-dione (fluorescamine) reacts directly with primary amines to form highly fluorescent products.

Several factors make fluorescamine suitable for assaying primary amines, including amino acids, peptides and proteins. At pH 8-9, the reaction with primary amines proceeds at room temperature (140) within a fraction of a second. Excess reagent is concomitantly destroyed within several seconds (140). Fluorescamine, as well as its hydrolysis products, is nonfluorescent. Studies with small peptides have shown that the reaction goes to near completion (about 80% to 95% of theoretical yield) even when fluorescamine is not present in excess. The following is an example of the reaction of fluorescamine with an amine group illustrating the product formed (fluorophor) and the rate of the reaction (100-500 msec). In addition, the reaction of water with fluorescamine with the formation of a nonfluorescent product is also shown.

Fluorescamine
(nonfluorescent)

+ RNH₂

+ H₂O

t ½
100–500 msec

t ½
5–10 sec

Fluorophor

Hydrolysis products
(nonfluorescent)
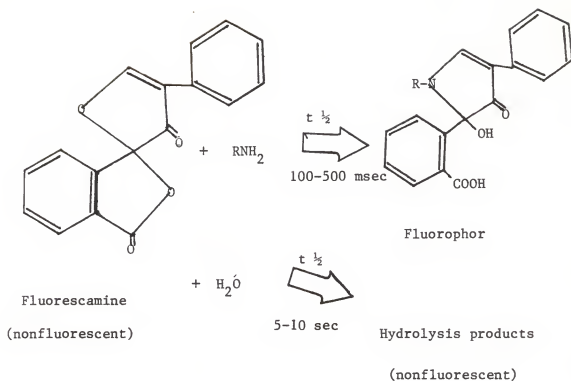
Primary amines are first buffered to an appropriate pH (7-8), and then fluorescamine, dissolved in a water miscible, nonhydroxylic solvent such as acetone or dioxane, is added. The reaction is complete, and in less than a minute excess reagent is destroyed. The resulting fluorescence is proportional to the amine concentration, and the fluorophors are stable over several hours. The above properties lend themselves well to automation (123). It should be noted that fluorescamine does not react with proline or hydroxyproline, which are not primary amines. This disadvantage can be overcome by introducing an appropriate intermediate step to convert these amino acids to primary amines (63,146). An additional advantage of the fluoresamine assay is that comparatively little fluorescence is developed with ammonia. Therefore, ammonia does not interfere with an analysis to the extent that it does in the colorimetric ninhydrin procedure. Figure 2 shows a comparison of the
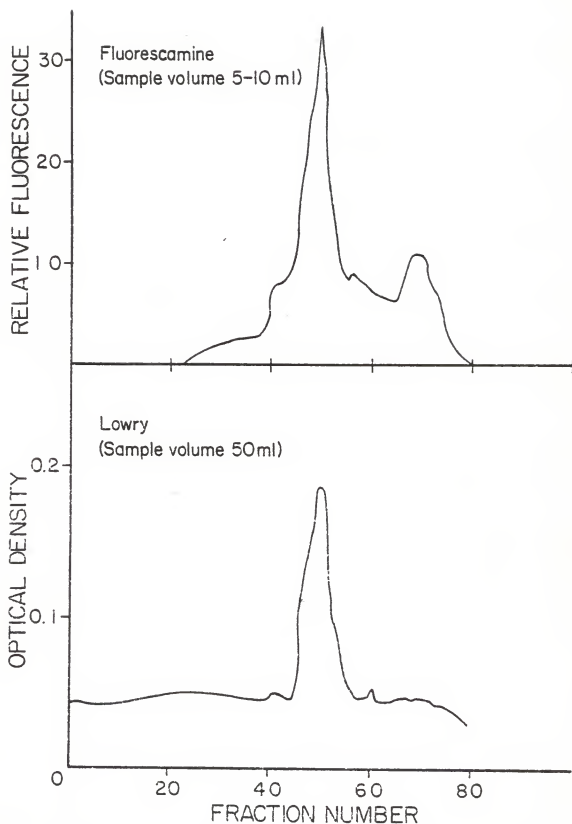
Figure 2. Comparison between the Fluorescamine technique and the Lowry procedure (140) for determining protein concentration. [a]
[a] Chromatography of a partially purified enzyme of guinea pig neurophysin monitored by the fluorescamine and the Lowry procedure.

fluorescamine technique with the standard Lowry procedure (97) for the monitoring of protein in a column effluent. The volumes used for fluorescamine assay were 10 to 20 percent of those used in the Lowry method, and smaller amounts could have been used (140). Background interference was negligible with the automated fluorescence method, and significant peaks not discernible by the Lowry procedure were observed.

Due to the many advantages of the relatively new fluorometric technique, it was used to measure the proteolytic enzyme activity of _P. citreus_. The ability of this organism to grow on shrimp as well as to hydrolyze various protein preparations promoted investigations to isolate, purify and characterize the extracellular enzyme(s) produced by _P. citreus_.

MATERIALS AND METHODS

Unless otherwise specified, Difco (55,56) or Baltimore Biological Laboratories (BBL) (16) products were used for all microbiological analyses. Serial dilutions used Butterfield's Phosphate buffer and followed the procedures outlined in the Compendium of Methods for the Microbiological Examination of Foods (6). All chemicals used were reagent grade meeting American Chemical Society specifications. All media and glassware were autoclaved for 15 min at 121 C unless label directions specified otherwise.

Planococcus citreus Cultures

The culture of P. citreus used in this study, A-17, was isolated from rock shrimp (Sicyonia brevirostris) (90). The culture chosen was able to grow well in shrimp during iced storage and showed strong proteolytic activity toward various protein preparations. The isolate used for the study was grown on Plate Count Agar slants (Difco) with 0.5% sodium chloride (NaCl) added and incubated at 20 C for 72 hrs (4). Appropriate dilutions in buffer were made to obtain a concentration of approximately 5×10^3 organisms per ml. The A-17 isolate used was capable of hydrolyzing gelatin, whey, cottonseed, soy, hog blood and shrimp protein preparations (4). It also grew well in 0.5% to 16% NaCl and pH 7.0 to 10.9.

Determination of Proteolytic Activity

A modified fluorescamine fluorescent (fluorometric) technique (129) was used to measure enzyme activity. Fluorescamine[R] is capable of

detection of amino acids, peptides, proteins and primary amines in the picomole range (140).

P. citreus cells were grown in various media throughout the study. After incubation, the cultures were centrifuged in a RC-5 Superspeed Refrigerated Centrifuge (Sorval, Dupont Co. Instruments, Newtown, CT) at a force of 20,000 x g for 30 min. The supernatant (cell-free broth) was used for further investigations involving extracellular enzymes. The cell pellet was washed twice with 0.05 M phosphate buffer (pH 8). The whole cells were then resuspended with 10-20 ml of the same buffer, transferred to a dry ice chilled Eaton pressure cell (60) and allowed to freeze under dry ice for 3 hrs. The frozen microbial cells were disintegrated using the Eaton pressure cell at a constant pressure of 7.03×10^{6} kg/m^{2} on a Carver hydraulic laboratory press (F. S. Carver, Inc., Summit, NJ). The ruptured cell extract was fractionated into intracellular soluble and particulate fractions by centrifugation at a force of 12,000 x g for 15 min. The particulate fraction was resuspended in 10 ml of 0.05 M phosphate buffer prior to enzyme activity determinations of all fractions. Five milliliters of the substrate (gelatin or shrimp protein) were reacted with 1 ml of each of the above fractions for 15 min at 35 C. The reactions were terminated by adding 10 ml of 5% TCA. Zero time blanks were prepared by adding the trichloroacetic acid (TCA, Fisher Scientific Co., Fairlanes, NJ) before the incubation period (see latter part of this section).

One milliliter of the cell-free broth or 100 µl of the purified enzyme was reacted with 5 ml of substrate (gelatin or shrimp protein) for the appropriate reaction time (to be determined) at 35 C, pH 8. The enzyme-substrate reaction was stopped by precipitating the mixture with

10 ml of 5% TCA. After 5 to 10 min, to allow the proteins to settle, the solution was filtered through Whatman #1 filter paper. Two hundred microliters of the TCA filtrate were transferred to a 13 x 100 mm test tube (Dispo culture tubes, Scientific Products, McGraw Park, IL) and the volume brought to 1.5 ml with 0.5 M sodium phosphate buffer, pH 8. While the test tube was vigorously mixed in a Vortex Mixture (Scientific Products, Evanston, NY), 0.5 ml of fluorescamine in dioxane (30 mg/100 ml, Eastman Kodak Corp., Rochester, NY) was rapidly added to the buffered protein solution. A model 204-A Fluorescence Spectrophotometer (Perkin Elmer Corp., Norwalk, CT) was used to measure fluorescence intensity. Zero time blanks were prepared by adding 10 ml of 5% TCA after adding the enzyme and prior incubation of the mixture. This blank represented the background activity present in the mixture at zero time. Zero time fluorescence reading was subtracted from the reading of the substrate-enzyme mixture after the appropriate incubation time.

Total enzyme activity was expressed as the change in 0.1 fluorescence units of the TCA filtrate per milliliter of enzyme per minute. Specific activity was expressed as the units of total enzyme activity/mg of protein present (units of activity/mg of protein).

Previous research involving the use of the fluorescamine technique (47,127,140) indicated that pH affected fluorescence intensity. Buffers of pH from 2 to 10 (see buffers described on pg. 44) were used to determine the effect of varying the pH of the buffer on fluorescence intensity. TCA filtrates (0.2 ml) were reacted with 1.3 ml of the various buffers (pH 2–pH 10) before addition of the fluorescamine reagent. Figure 3 indicates that addition of pH 8 buffer resulted in the highest fluorescence itensity. Consequently, pH 8 buffer was used for the remainder of the research.
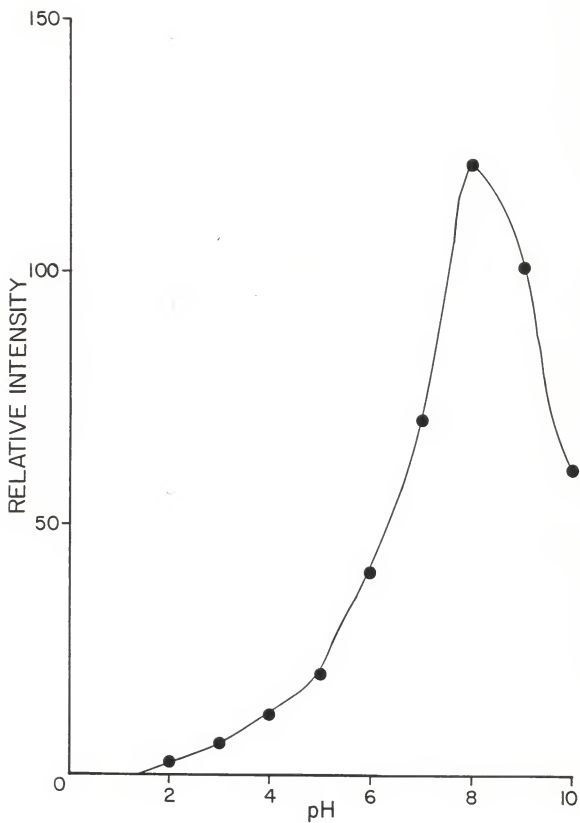
Figure 3. Effect of pH adjustment of gelatin-trichloroacetic acid (TCA) filtrates on fluorescence intensity.

In order to determine the reaction spectrum of our working enzyme solution when gelatin and shrimp protein were used as substrates, the excitation and emission (fluorescent) wavelengths were scanned (48). Figure 4 shows the excitation (curve A) and fluorescence (curve B) spectra for TCA filtrates of the gelatin substrate. The excitation spectrum has a maximum of 360 nm and a secondary peak at 390 nm. The secondary peak at 390 nm was chosen because it results in minimal zero time blank fluorescence values. The fluorescence emission maximum with the excitation wavelength at 390 nm was at 475 nm. Figure 5 shows the excitation (curve A) and fluorescence (curve B) emission spectra for TCA filtrates of the shrimp protein substrate. The excitation spectrum has a maximum peak at 375 nm and a secondary peak at 390 nm. Again, the secondary peak was chosen. The fluorescence emission maximum with the excitation wavelength at 390 nm was at 490 nm.

Efficacy of 5% Trichloroacetic Acid (TCA)

In order to determine the efficacy of 10 ml of 5% trichloroacetic acid (TCA) in terminating the enzyme-substrate reaction, 5 ml aliquots of substrate (gelatin) were incubated with 1 ml of cell-free broth and 10 ml of 5% TCA for 0, 10, 15, 30, 45 and 60 min at 35 C. A positive control was done by incubating the enzyme-substrate mixture at 35 C for 0, 10, 15, 30, 45 and 60 min before adding the TCA. After the incubation period, the positive control was terminated by adding 10 ml of 5% TCA. Data in Figure 6 shows that 10 ml of 5% TCA were adequate for inhibiting the enzyme substrate reaction effectively since there was no increase in fluorescence intensity. The fluorescence intensity, observed when TCA is immediately reacted with the enzyme and substrate, represents the background fluorescence of the assay mixture.
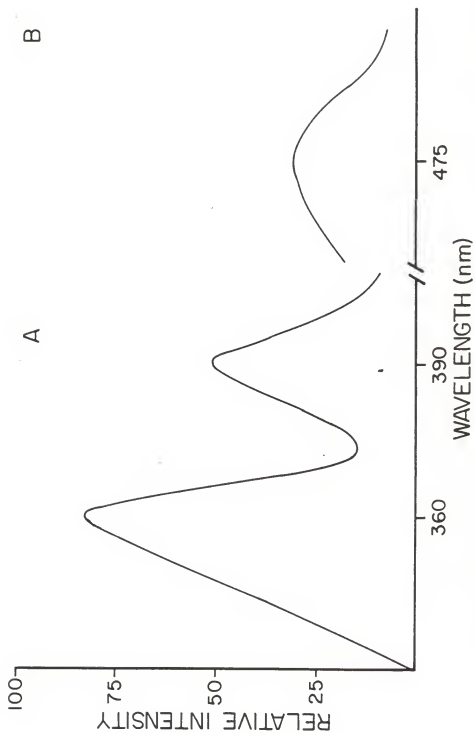
Figure 4. Exitation (curve A) and fluorescence (curve B) spectrum for the reaction of a gelatin-trichloro-acetic acid (TCA) filtrate with fluorescamine at pH 8.
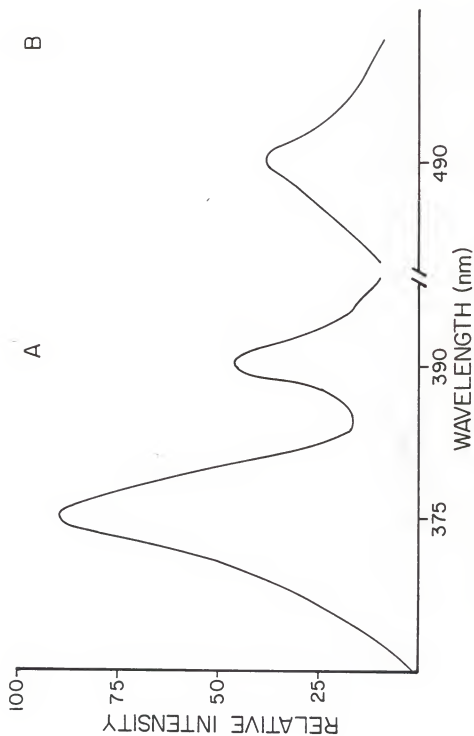
Figure 5. Exitation (curve A) and fluorescence (curve B) spectrum for the reaction of a shrimp protein-trichloroacetic acid (TCA) filtrate with fluorescamine pH 8.
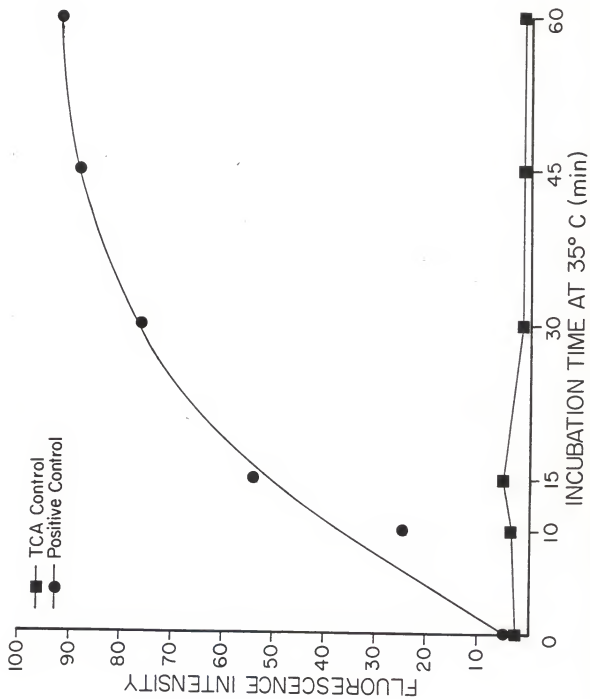
Figure 6. Efficacy of trichloroacetic acid (TCA) in terminating the enzyme-substrate reaction.

Substrate Characteristics

Two substrates were used throughout the study: gelatin (Difco) (1.2 mg/ml) and a shrimp protein preparation (0.6 mg/ml). Higher concentrations of the shrimp protein preparation were not used because of solubility problems in the buffer systems used. The shrimp protein preparation was prepared as follows: fresh raw shrimp meat ground in a Waring blender with 0.05 M phosphate buffer (pH 7, 1:10 dilution), dialyzed overnight with four changes of the same buffer at 5 C and lyophylized for preservation (Virtis Freeze Dryer, Gardiner, NY). Protein, fat, moisture and ash were determined for the shrimp protein preparation. Protein was determined by the AOAC standard micro-Kjeldahl method (13). Crude fat was determined by a modification of the AOAC method (13) using the Goldfisch solvent chamber. Approximately 2 grams of sample were extracted overnight with petroleum ether. Moisture was determined in a vacuum oven at 70 C for 12 hrs. Ashing was done in a muffle furnace at 600 C for 8 hrs. Table 3 shows that the shrimp protein preparation consisted of 77.44% protein, 5.40% fat, 8.95% moisture, 6.50% ash and 1.71% carbohydrate (calculated by difference).

Determination of Enzyme-Substrate Mixture Reaction Time

Five milliliters of substrate and an aliquot of cell-free broth were incubated at 35 C for 0, 5, 10, 15, 20, 30 and 60 min in order to determine the time course of enzyme activity and apparent optimum reaction time. In experiments involving gelatin, 0.5, 1.0 and 2.0 ml of cell-free broth were used while 1.0 ml of cell-free broth was used with the shrimp protein substrate. An incubation time of 15 min was an appropriate enzyme-substrate contact reaction time when shrimp were used as substrate (Figure 7). In addition, when gelatin was used as a substrate and

Table 3. Proximate composition of the shrimp protein preparation.[a]

	Percent (%)
Protein	77.44
Fat	5.40
Moisture	8.95
Ash	6.50
Carbohydrate[b]	1.71

[a]Average of two determinations

[b]Calculated by difference
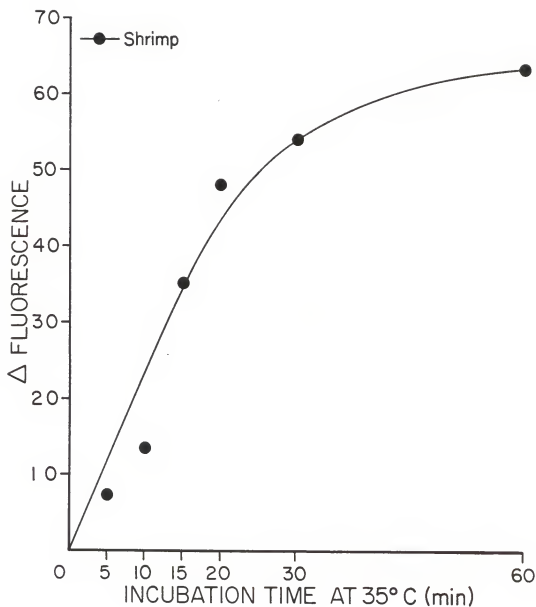
Figure 7. Increase in fluorescence intensity using the shrimp protein preparation as substrate after incubation with cell-free broth for up to 1 hr at 35 C.

various amounts of cell-free broth were reacted with gelatin, a 15 min reaction time was also a sufficient substrate reaction time (Figure 8). This reaction time was used for the remainder of the study.

Growth Medium and Enzyme Production

Various media were used to determine growth rates and production of extracellular enzyme(s) by $\underline{P}$. $\underline{citreus}$. Three hundred milliliters of Plate Count Broth (PCB) + 0.5% NaCl, Nutrient Broth (NB) + 0.5% NaCl and Trypticase Soy Broth (TSB) were used to grow the organism. Incubation was at 20 C for up to 96 hrs. Samples were drawn at 0, 12, 24, 48, 72 and 96 hrs. All samples were assayed for growth by measuring optical density at 600 nm in a Spectronic-20 Spectrophotometer (Bausch and Lomb, Rochester, NY) and by plating in Plate Count Agar (PCA) with incubation at 20 C for 5 days. Cultures were centrifuged in a RC-5 Superspeed Refrigerated Centrifuge at a force of 20,000 x g for 30 min. The sediment was discarded. One milliliter of the cell-free broth was assayed for enzyme activity with gelatin substrate. $\underline{P}$. $\underline{citreus}$ growth and enzyme analyses were done three times and each time in duplicate.

Optimization of Enzyme Activity to Growth and Cell Number

The optimum time for cell harvesting along the logarithmic section of the growth curve (approximately midlog) of the organism was selected. The logarithm of the cell count was plotted against the incubation time. The specific growth rate (k) of each medium was calculated using the equation $\ln B_x = \ln B_o + k^t$ (where B_x = bacterial count at time x, B_o = bacterial count at time 0 (both in midlog growth phase), k = specific growth rate, t = time, hrs). The units of enzyme activity per cell per hour (m) were then calculated using the formula (58,124): $M_t - M_o = \frac{m}{k}$ $(B_x - B_o)$ (where M_t = enzyme activity at cell number B_x, M_o = enzyme
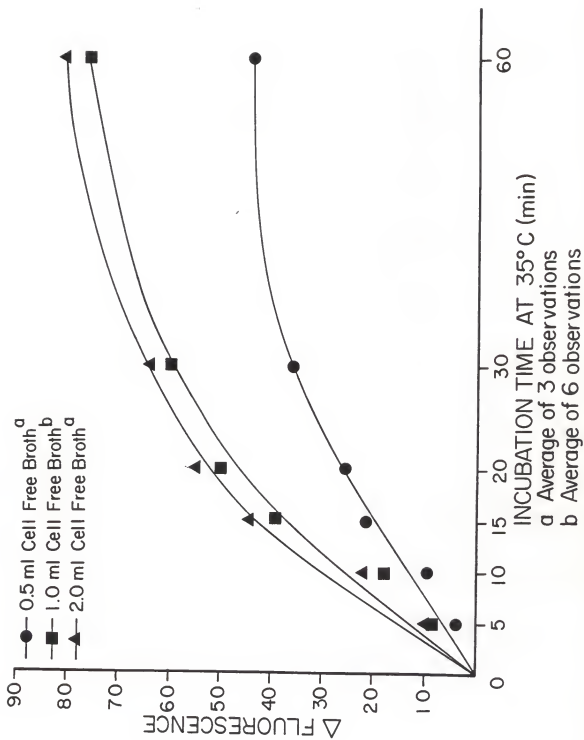
Figure 8. Increase in fluorescence intensity using gelatin as substrate and various amounts of the cell-free broth after incubation at 35 C for 1 hr.

activity at cell number B_o, k = specific growth rate). This formula was obtained through the integration of the following equation: $\frac{dm}{dt} = m \, B_o \, e^{kt}$ (where dm/dt = change in enzyme activity over time). The calculated m's were compared for the three media used. A test to observe any difference between the values for the units of enzyme activity per cell per hour (m) in each medium was designed using the Statistical Analysis System (SAS) program package (15) for analysis of variance. A completely randomized design (102,132) was used in that the major source of error to be considered was due to the nutritional differences between media.

Effect of Incubation Temperature on Enzyme Production and Activity

The effect of incubation temperature (5, 20 and 35 C) on the growth of P. citreus and its ability to produce an active extracellular enzyme was investigated. Five-hundred-milliliter Erlenmeyer flasks containing 100 ml of medium were inoculated with approximately 5×10^3 P. citreus and incubated at the three temperatures. All samples were assayed for growth by measuring optical density at 600 nm in a Spectronic-20 spectrophotometer and for proteolytic activity using the fluorometric technique. For the 35 C grown cells, samples were drawn at 0, 6, 12, 24, 48, 72 and 96 hrs. For the 20 C grown cells, samples were drawn at 0, 12, 24, 48, 72, 96 and 120 hrs. Finally, for the 5 C grown cells, samples were drawn at 0, 24, 48, 72, 96, 120 and 144 hrs. After determining the midlog phase of growth for P. citreus at each temperature, P. citreus cells were then harvested at this stage. Enzyme activity determinations were done using the cell-free broth obtained from growing the organism at the three temperatures until midlog phase. Five milliliters of the shrimp protein preparation were incubated with 1 ml of each cell-free broth at 5 C for 60 min, 20 C for 30 min and 35 C for 15 min. Analyses were done three times and each time in duplicate.

In addition, direct microscopic observations of the cells grown at 5, 20 and 35 C were conducted. Any morphological change due to growth temperature was observed and recorded.

Purification of the Extracellular Enzyme(s)

Planococcus citreus was grown in the selected medium at 20 C until midlog phase. The cells were then centrifuged at a force of 20,000 x g for 30 min. This cell-free broth was used in the purification procedure.

Ammonium Sulfate Precipitation

Fractional precipitation of the enzyme(s) in the cell-free broth was accomplished with 0-55%, 55-70% and 70-100% ammonium sulfate saturation (Mallinckrodt, Inc., Paris, KY). The required amount of ammonium sulfate was added with stirring until dissolved (88). The mixture was allowed to equilibrate for 1 hr at 4 C and centrifuged at a force of 20,000 x g for 20 min. The precipitate was resuspended with 10 ml of Butterfield's phosphate buffer at pH 8 and dialyzed for 16 hrs (dialysis tubing #24, Scientific Products, McGraw Park, IL) against 500 ml of 0.05 M phosphate buffer pH 8 (108,153). Each ammonium sulfate fraction was then assayed for proteolytic activity using gelatin as substrate.

Molecular Sieve Chromatography

Sephacryl[R] S-200 Superfine (Pharmacia Fine Chemicals, Uppsala, Sweden), a high resolution chromatographic medium for gel filtration was used to separate the enzyme solution according to molecular weight after the ammonium sulfate precipitation step. A 30 x 2.5 cm column was packed with Sephacryl[R] S-200 Superfine gel and a Pharmacia[R] peristaltic pump (p-3) was used to pack the column at a speed of 40 ml/cm/hr (120). The enzyme solution was eluted using reverse flow at a speed of 30 ml/cm/hr. Five milliliter fractions were collected in each tube with 0.02 M phos-

phate buffer pH 7 as the eluting agent using a Gilson Fraction collector
(Model FC-220K Fractionator, Gilson Medical Electronics, Inc., Middle-
town, WI). Enzyme activity of each fraction collected was then measured
using gelatin as substrate. The protein present in the fractions was
determined by following absorbance at 280 nm using a Beckman Model 25
spectrophotometer (Beckman Instruments, Inc., Fullerton, CA).

Ion-Exchange Chromatography

The active fractions recovered from the gel filtration step were
pooled and further separated by ion-exchange chromatography using DEAE-
Sephadex[R] A-50 (Pharmacia Fine Chemicals, Uppsala, Sweden). The column
was prepared following the procedures given by Pharmacia Fine Chemicals
(119). A 40 x 2.5 cm column was prepared and the protein eluted with
0.02 M phosphate buffer with a linear gradient of tris 0.01 M NaCl-tris
0.15 M NaCl (47) at a rate of 25 ml/hr (1.5 reading in the peristaltic
pump) and collected in 10 ml fractions. Two hundred fifty milliliters
of 0.01 M and 0.15 M NaCl solutions were placed in each vessel for the
linear gradient. The protein present in the fractions was followed by
reading the absorbance at 280 nm using a Beckman Model 25 spectrophotom-
eter. Enzyme activity of each fraction was measured with gelatin as the
substrate. All column studies were duplicated.

The protein content of each fraction eluted using the Sephacryl[R]
S-200 Superfine and the DEAE-Sephadex[R] A-50 was also analyzed by the
Lowry (98) method for protein with Bovine Serum Albumin (Sigma Chemical
Co., St. Louis, MO) as the standard. Protein content is expressed as
mg/ml. Figure 9 summarizes the steps followed in the purification of
the extracellular proteolytic enzyme(s) of P. citreus.
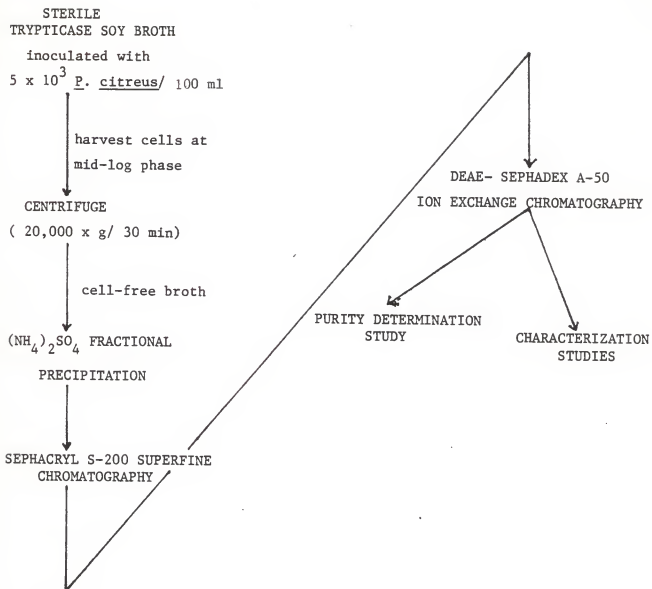
Figure 9: Outline of steps for the purification of the extracellular protease(s) of Planococcus citreus.

Confirmation of Enzyme Purity

A modification of the Weber and Osborn (144) method for sodium dodecyl sulfate-poly acrylamide gel (SDS-PAG) gel electrophoresis was used. A Buchler 3-1500 electrophoresis apparatus (Buchler Instruments Corp., Fort Lee, NJ) was used to evaluate the purity of the isolated extracellular enzyme.

A 10% acrylamide:BIS, 30:0.8 gel was prepared and allowed to poly-merize for 2 hrs. A sample of the purified enzyme was diluted 1:1 with the sample buffer. The sample buffer consisted of 0.01 M sodium phos-phate (pH 7), 10% sodium dodecyl sulfate, 0.1% dithiothrietol, 10% glyc-erol and 0.001% bromocresol blue. The protein solutions were placed onto the gels (50 µg protein/gel, 100 µg/gel, 150 µg/gel and 200 µg/gel) and were layered carefully with electrode buffer (pH 8.3) to the top of each tube. The lower electrode chamber was then 2/3 filled with electrode buffer. The tubes in the apparatus were then lowered into the electrode chamber. The upper chamber was filled with water to approximately 1 inch over the tube top. The water jacket was connected and the electrode wires from the power source were also connected. A constant current of 1-1.5 mAmps/gel was applied until the marker dye band just exited from the gels (approximately 3 hrs). The gels were immediately removed from their tubes. The gels were fixed overnight in a fresh 50% TCA solution. The fixed gels were then stained 1-2 hrs with 0.1% Coomassie brilliant blue solution made up fresh in 50% TCA at 37 C in a water bath. The gels were further diffusion-destained by repeated washings in 7% acetic acid (17-72 hrs). Gels were then stored in 7% acetic acid (82).

Characterization of the Proteolytic Enzyme(s)

Molecular Weight Determination

Two methods (7,21,150) were used to estimate the molecular weight of the enzyme(s).

A 2.5 x 30 cm column packed with Sephacryl[R] S-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) was used and the following standards applied: Ribonuclease A (13,000 MW), Trypsin (23,500 MW), Pepsin (45,000 MW), Bovine Serum Albumin (70,000 MW) and Aldolase (158,000 MW) (Pharmacia Fine Chemicals, Piscataway, NJ) following the procedures suggested by Pharmacia Fine Chemicals (116). The K_{av} value of each protein sample was calculated and plotted against the corresponding molecular weight ($K_{av} = \frac{V_e - V_o}{V_t - V_o}$ where V_o = column void volume, V_e = elution volumes and V_t = total column volume). Protein was monitored at 280 nm using a Beckman Model 25 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA).

Bio-Rad[R] Low Molecular Weight Protein Standards (10,000-100,000) for SDS Gel Electrophoresis were also used for molecular weight determination using SDS-PAG gel electrophoresis. The instructions outlined by Bio-Rad[R] (Bio-Rad Laboratories, Richmond, CA) were followed (21). The proteins included were Phosphorylase B, Bovine Serum Albumin, Ovalbumin, Carbonic Anhydrase, Soybean Trypsin Inhibitor, Lysozyme and the purified enzyme. The motility of the enzyme was then compared to the relative motility (Rm) of the standards.

Determination of the Purified Enzyme-Substrate Mixture Reaction Time

Five milliliters of substrate (gelatin or shrimp protein) and 100 µl of the purified enzyme were incubated at 35 C for 0, 5, 10, 15, 30 and 60 min in order to determine the apparent optimum reaction time.

Enzyme activity was measured using the Fluorescamine technique. Figure 10 shows that 10 min was the optimum reaction time for the purified enzyme-substrate (gelatin or shrimp protein) reaction mixture. This optimum reaction time was used for the remainder of the characterization of the extracellular proteolytic enzyme.

Effect of Ionic Strength on Enzyme Activity

The effect of ionic strength on enzyme activity was investigated. Gelatin (1.2 mg/ml) was dissolved in the following solutions of sodium chloride (NaCl): 0.05 M (μ = 0.13), 0.08 M (μ = 0.16), 0.18 M (μ = 0.26), 0.25 M (μ = 0.35), 0.34 M (μ = 0.42), 0.51 M (μ = 0.59), 0.75 M (μ = 0.83), 1.00 M (μ = 1.08) and 1.5 M (μ = 1.58). The NaCl was dissolved in 0.05 M phosphate buffer (pH 8). Five milliliters of this mixture were reacted with 100 μl of the purified enzyme and incubated at 35 C for the selected reaction time (10 min).

Determination of Optimum pH

Buffers of varying pH from pH 2 to pH 10 were used to determine the optimum pH for the proteolytic activity of the enzyme(s). The following buffers were used:

		Ionic Strength
pH 2	0.1 M citric acid	0.25
3	47.0 ml of 0.1 M citric acid + 3.5 ml of 0.1 M sodium citrate	0.35
4	33.0 ml of 0.1 M citric acid + 17.0 ml of 0.1 M sodium citrate	0.45
5	20.5 ml of 0.1 M citric acid + 29.5 ml of 0.1 M sodium citrate	0.45
6	88 ml of 0.2 M monobasic sodium phosphate + 12.5 ml dibasic sodium phosphate	0.45
7	39.0 ml of 0.2 M monobasic sodium phosphate + 61.0 ml of 0.2 M dibasic sodium phosphate	0.35
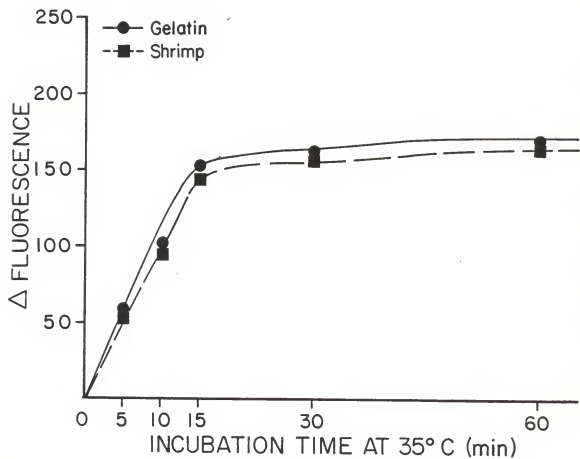
Figure 10. Increase in fluorescence intensity following incubation of gelatin and shrimp protein substrate with purified enzyme for up to 1 hr at 35 C.

		Ionic Strength
8	5.3 ml of 0.2 M monobasic sodium phosphate + 95.0 ml of 0.2 M dibasic sodium phosphate	0.25
9	50 ml of 0.2 M glycine + 8.8 ml of 0.2 M NaOH	0.22
10	50 ml of 0.2 M glycine + 32.0 M NaOH	0.20

Gelatin (1.2 mg/ml) or shrimp protein (0.6 mg/ml) were dissolved in the various buffers. Any pH adjustments due to the addition of the substrates were done using 10 mM HCl or 10 mM NaOH. Five milliliters of this mixture were reacted with 100 µl of the purified enzyme and incubated at 35 C for the selected reaction time (10 min).

Determination of Optimum Temperature

Five milliliters of gelatin or shrimp protein substrate and 100 µl of the purified enzyme were incubated at 5, 10, 20, 35, 45, 55 and 65 C for 10 min at the optimum pH determined in the previous section.

Thermal Stability

P. citreus was incubated at 5 and 35 C in 300 ml of Trypticase Soy Broth (TSB). Cell-free broths obtained at midlog phase, 108 and 36 hrs for the 5 and 35 C grown cells, respectively, were used in this study. Five milliliters of the cell-free broths were incubated at 35, 45, 55, 65, 75 and 85 C for 15 min. The heat treated cell-free broths solutions were rapidly cooled (87), and their activity was assayed at 35 C for 15 min using gelatin as substrate. The residual activities at each solution were compared to the activity observed when the cell-free broths were incubated with the substrate at 35 C for 15 min.

In addition, 1 ml of the purified enzyme was also incubated at 35, 45, 55, 65, 75 and 85 C for 10 min. The heat treated purified enzyme solution was cooled, and its activity assayed at 35 C for 10 min using

gelatin as substrate. The residual activities of each solution were compared to the activity observed when the purified enzyme was incubated with gelatin at 35 C for 10 min.

Effect of Sodium Chloride Concentration

Various concentrations of NaCl were tested for their effect on enzyme activity. Concentrations of 0.00, 0.25, 0.50, 0.75, 1.00, 1.25 and 1.50% were used. NaCl was dissolved in 0.05 M phosphate buffer pH 8. The shrimp protein (0.6 mg/ml) and gelatin (1.2 mg/ml) were dissolved in the NaCl solutions. Five milliliters of the NaCl solutions were incubated with 100 μl of the purified enzyme at 35 C for 10 min.

Effect of Sodium Bisulfite Concentration

Various concentrations of sodium bisulfite ($NaHSO_3$) were tested for their effect on enzyme activity. Concentrations of 0.0, 0.5, 1.0, 2.0 and 3.0% were tested. $NaHSO_3$ (J. T. Baker Chemical Co., Phillipsburg, NJ) was dissolved in 0.05 M phosphate buffer pH 8. The shrimp substrate was dissolved in these $NaHSO_3$ solutions (0.6 mg/ml). Five milliliters of the $NaHSO_3$ solutions were incubated with 100 μl of the purified enzyme at 35 C for 10 min.

Effect of Enzyme Concentration

Various quantities of enzyme (from 0 to 200 μl) were tested to observe the effect of enzyme concentration on enzyme activity. Five milliliters of substrate (gelatin or shrimp protein) were incubated with 0, 50, 75, 100 and 200 μl of enzyme at 35 C for 10 min.

Effect of Substrate Concentration

The enzyme was incubated with various concentrations of gelatin and shrimp protein in order to determine substrate saturation conditions. For the gelatin substrate, 0.00, 0.15, 0.30, 0.45, 0.60 and 1.20 mg/ml

were tested. However, for the shrimp substrate, 0.000, 0.075, 0.100, 0.125, 0.150, 0.300 and 0.600 mg/ml were tested. Five milliliters of each substrate solution were reacted with 100 µl of the purified enzyme at 35 C for 10 min. From these data, Lineweaver-Burk plots were derived, and K_m and V_{max} values for each substrate were extrapolated from these plots (95,152).

Effect of Metal Ions on Enzyme Activity

Calcium chloride (10, 20 mM), ferric chloride (1, 20 mM), magnesium chloride (10, 20 mM), mercurous chloride (1, 20 mM), zinc chloride (10, 20 mM), manganese chloride (10, 20 mM) and potassium chloride (5, 20 mM) were tested for their effect on enzyme activity (all metals were dissolved in 0.05 tris-HCl buffer). For the control, a buffer with no metal ions added was used (76,87). Five milliliters of substrate (gelatin) 100 µl of enzyme and 1 ml of the metal ion buffer solution were reacted for 10 min at 35 C. The fluorometer reading of the control sample was compared to the reading of the metal ion samples.

Effect of Various Reagents on Enzyme Activity

Ethylene diaminetetraacetic acid (EDTA) (10, 20 mM), citric acid (10, 20 mM), formaldehyde (1, 20 mM), potassium cyanide (KCN) (1, 20 mM), potassium permanganate ($KMnO_4$) (1, 20 mM), cysteine (1, 20 mM), 2-mercaptoethanol (1, 20 mM), p-dioxane (10, 20 mM) and trichloroacetic acid (TCA) (5, 10%) were tested for their effect on the proteolytic activity of the P. citreus enzyme (76,87). All reagents were dissolved and/or mixed with 0.05 M tris-HCl buffer. Five milliliters of substrate (gelatin), 100 µl of enzyme and 1 ml of the appropriate reagent buffer solution were reacted at 35 C for 10 min. A control with no reagent added was used and the fluorometer reading from the various reagents was compared to the control.

Dipeptidase Activity

The potential of the P. citreus enzyme to degrade peptides was investigated. DL-leucylglycine, DL-leucyl-DL-alanine, glycyl-DL-leucine, DL-alanylglycine and L-leucyl-l-tryptophan (Sigma Chemical Co., St. Louis, MO) were used in this study. Fifty milligrams of each dipeptide were dissolved in 50 ml of phosphate buffer, pH 8. Five milliliters of the dipeptide solutions were incubated with 100 µl of the purified enzyme at 35 C for 10 min. The reaction was terminated by adding 10 ml of 5% TCA. Zero time blanks were done by adding the TCA to the enzyme-peptide mixture before the incubation period.

Enzyme Induction Studies

P. citreus was grown in various media in order to determine if the extracellular proteolytic enzyme produced by this organism is induced by shrimp protein. Three-hundred milliliters of the following were used:

(1)   Yeast Carbon Base (YCB) (control)

(2)   YCB + 1.0% Shrimp Protein

(3)   YCB + 0.1% Yeast Extract

(4)   YCB + 0.1% Yeast Extract + 1.0% Shrimp Protein

Table 4 shows the composition of the Yeast Carbon Base medium (YCB).

P. citreus growth and enzyme activity were analyzed at 0, 24, 48, 72 and 96 hrs following incubation at 20 C. Cell numbers were determined by pour plating into Trypticase Soy Agar (TSA) with incubation at 20 C for 5 days. Five milliliters of the shrimp substrate were incubated with 1 ml of the cell-free broth from each culture for 15 min at 35 C. The reaction was terminated by adding 10 ml of 5% TCA. Zero time blanks were done by adding the TCA to the cell-free broth-substrate mixture before the incubation period. This study was done twice in duplicate.

Table 4. Composition of yeast carbon base medium (56).

Formula in Grams per Liter of Distilled Water	
Boric Acid	0.500 mg.
Copper Sulfate	0.040
Potassium Iodide	0.100
Ferric Chloride	0.200
Manganese Sulfate	0.400
Sodium Molybdate	0.200
Zinc Sulfate	0.400
Biotin	0.002 mg.
Calcium Pantothenate	0.400
Folic Acid	0.002
Inositol	2.000
Niacin	0.400
p-Aminobenzoic Acid	0.200
Pyridoxine	0.400
Riboflavin	0.200
Thiamine HCl	0.400
L-Histidine HCl	0.001 g.
DL-Methionine	0.002
DL-Tryptophan	0.002
Potassium Phosphate	1.000 g.
Magnesium Sulfate	0.500
Sodium Chloride	0.100
Calcium Chloride	0.100
Dextrose	10.000
Final pH of the base adjusted to 7.5	

The data was analyzed in a similar manner as the Growth Medium and Enzyme Activity data. Again, a comparison of the m's for each medium used (m = units of enzyme activity/cell/hr) was attempted using the SAS program package for analysis of variance (15).

The Duncan's New Multiple-Range Test (pg. 187-190 (132)) was used to compare any difference in the calculated means of the data obtained after analysis of variance in the "Optimization of enzyme activity to growth and cell number" section (pgs. 36 and 38), "Optimum pH determination" section (pg. 82), "Optimum temperature determination" section (pg. 84) and "Enzyme induction study" section (pg. 105-115). The Duncan's New Multiple-Range Test was done using the SAS program package (15).

RESULTS AND DISCUSSION

The ability of _Planococcus_ _citreus_ to grow in shrimp during ice
storage raised the question as to whether this organism could contribute
to the spoilage of shrimp. Various studies (3,4,5) have indicated that
this organism may contribute to the spoilage of this valuable marine
resource. In order to more clearly understand the contribution this
organism makes to the degradation of shrimp, an investigation was under-
taken to study the extracellular proteolytic enzyme(s) produced by this
organism.

Proteolytic Activity of Cellular Fractions

The proteolytic activity of cellular fractions of _P._ _citreus_ cells
grown in Trypticase Soy Broth (TSB) was investigated in order to deter-
mine the distribution of the enzyme activity in the isolated fractions.
In addition to the cell-free broth (extracellular fraction), whole cells,
washings of the whole cells, soluble intracellular and the cellular
particulate fraction were examined. Table 5 shows the total activity
(units of activity), protein content (mg/ml), specific activity (units
of activity/mg of total protein) and distribution of activity (%) for
all the fractions tested using both gelatin and shrimp protein sub-
strates. The extracellular fraction showed the highest specific activ-
ity, 29.450 units of activity/mg of protein and 27.540 units of activ-
ity/mg of protein towards gelatin and shrimp protein, respectively.
This represented 95.9 and 95.8% of the total activity present in all of
the fractions towards gelatin and shrimp protein, respectively.

Table 5. Proteolytic activity at 35 C for 15 min (pH 8) of cellular fractions obtained from _Planococcus citreus_ grown in Trypticase Soy Broth (TSB) using gelatin and shrimp as substrates.

Fractions	Total Enzyme Activity (units)		Protein[a] (mg/ml)	Specific Activity[b] (units/mg total protein)		Distribution of Activity (%)	
	Gelatin	Shrimp		Gelatin	Shrimp	Gelatin	Shrimp
Whole cells	386.7	395.6	23.40	0.136	0.139	0.4	0.5
Extracellular	131,100.0	122,590.0	44.50	29.450	27.540	95.0	95.8
1st washing	251.1	253.3	6.26	0.401	0.406	1.3	1.4
2nd washing	163.3	133.3	3.26	0.500	0.408	1.6	1.4
Intracellular	101.1	81.1	10.33	0.098	0.079	0.4	0.3
Particulates	171.1	192.2	11.26	0.152	0.171	0.6	0.6

[a]Average of duplicate samples
[b]Total activity/Total protein = specific activity (units of activity/mg protein)

The whole cell fraction (cell bound fraction), both whole cell washings (loosely bound to cell wall fraction) and the particulate fraction exhibited low specific activity towards both high molecular weight substrates. The intracellular soluble fraction (the soluble fraction after the differential centrifugation of ruptured cells) exhibited the lowest specific activity when gelatin and shrimp protein were used as substrates (Table 5). These results show that the major portion (>95.0%) of the active enzyme towards these two high molecular weight substrates resides in the extracellular fraction.

Most microorganisms can synthesize various enzymes within their cell structure. Each enzyme system may have its own unique characteristics, and these characteristics will vary depending on the enzyme, the substrate and conditions during the enzyme-substrate reaction. In addition, the location of the proteinase(s) within the bacterial cell may vary markedly between microorganisms. Various researchers (53,115,135, 139) have studied the location of particular bacterial proteinases within the cell and how this location relates to the function of the enzyme. Thomas et al. (139), using gentle procedures for cell fractionation, suggested two criteria for the location of a proteinase produced by Streptococcus lactis. The two criteria they suggested were: 1) intact cells (whole cells) possessed substantial proteinase activity when incubated with a high molecular weight substrate; 2) most of the cell-bound proteinase activity was released during spheroplast formation. The solubilized cell wall, plasma membrane and cytoplasm fractions contained 84%, 0% and 16% activity, respectively, of the total proteinase activity with casein as substrate (139). In the results presented in this dissertation, the whole cell and cellular particulate fractions of

the P. citreus cells showed little enzyme activity towards gelatin and shrimp protein (both high molecular weight substrates).

Thomas et al. (139) also concluded that the cell wall proteinase may serve a similar nutritional role in nature as the surface-bound proteinases discussed by Payne and Gilvarg (115) and Sussman and Gilvarg (135). Gilvarg and his co-workers stated that surface-bound proteinase(s) appear to serve a nutritional role by hydrolyzing proteins to amino acids or peptides that are small enough to enter the cell. In turn, Payne, Sussman and Gilvarg (115,135) also suggested that the intracellular peptidases could further hydrolyze the peptides formed and release their constituent amino acids, thus, permitting the utilization of the protein substrate for growth. In Table 5, we can observe that certain P. citreus fractions (whole cells, intracellular and cellular particulate) had substantial amounts of protein present. Perhaps some of the protein present in these fractions include other enzymes (i.e., peptidases) that can utilize the peptides produced by the action of the extracellular protease(s) that later may enter the P. citreus cell. In this manner, P. citreus cells could fully utilize the protein available (i.e., shrimp protein as well as other proteins) for their growth.

Growth Medium and Enzyme Production

Trypticase Soy Broth (TSB), Plate Count Broth (PCB) + 0.5% NaCl and Nutrient Broth (NB) + 0.5% NaCl were used to determine growth rates and production of extracellular proteolytic enzyme(s) by P. citreus. Figure 11 shows the growth of P. citreus, as measured by the increase in optical density (600 nm), in the three media used. In all three media, P. citreus exhibited a 12 hr lag phase in which an increase in optical density was not evident. After this lag period, TSB supported the most
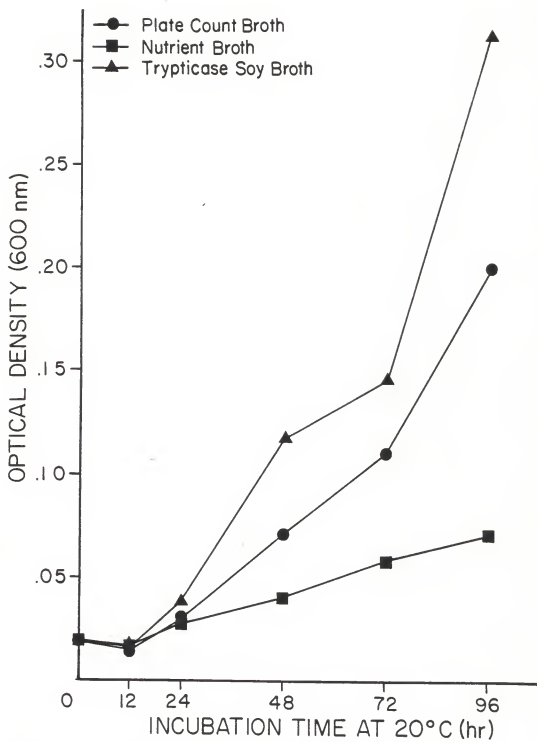
Figure 11. Spectrophotometric growth curves of _Planococcus citreus_ in Plate Count Broth, Nutrient Broth and Trypticase Soy Broth at 20 C.

rapid growth of P. citreus. The optical density after 96 hours of incubation at 20 C was .314, .200 and .072 for TSB, PCB and NB, respectively. Figure 12 shows a similar trend; however, P. citreus growth was measured by the Aerobic Plate Count technique (6). Again, we can observe that the P. citreus log count per ml increases slightly during the first 12 hours of incubation at 20 C. After 96 hrs of incubation, the P. citreus log count for TSB, PCB and NB was 6.02, 5.10 and 4.17, respectively. Consequently, TSB allowed for the "optimum" growth of P. citreus when grown at 20 C.

Nutritional components present in the growth medium are of utmost importance for gram-positive microorganisms which are generally more fastidious in its nutrient requirements than gram-negative bacteria (29). Realizing these growth requirements of gram-positive microorganisms, the results from this section are not surprising. TSB contains tryptone, soytone, dextrose, sodium chloride and dipotassium phosphate (16). This combination of nutrients provide an adequate nitrogen, carbohydrate, vitamin and overall nutrient supply for the growth of P. citreus. In contrast, PCB and NB are not as nutritionally complex.

Figure 13 shows the enzyme activity of the cell-free broth of P. citreus cells grown in TSB, NB and PCB for 96 hrs at 20 C. An active extracellular enzyme fraction was produced by P. citreus in all three media. However, after 96 hrs of growth, the amount of enzyme produced by this organism in TSB is greater than that produced when grown in PCB of NB. The enzyme activity after 96 hrs of incubation of the cell-free broth of P. citreus grown in TSB, PCB and NB was 323, 270 and 200 units of activity, respectively. However, if the cells are harvested at approximately midlog phase, the difference in the amount of enzyme
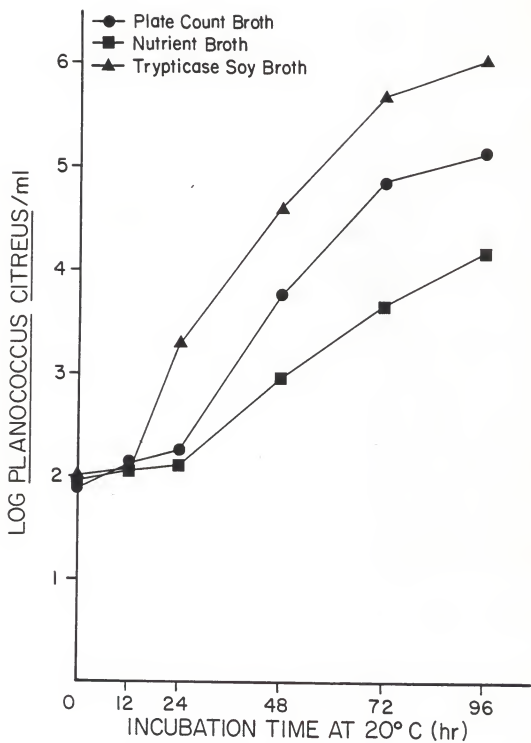
Figure 12. Aerobic plate counts of _Planococcus citreus_ incubated in Plate Count Broth, Nutrient Broth and Trypticase Soy Broth at 20 C for 96 hrs.
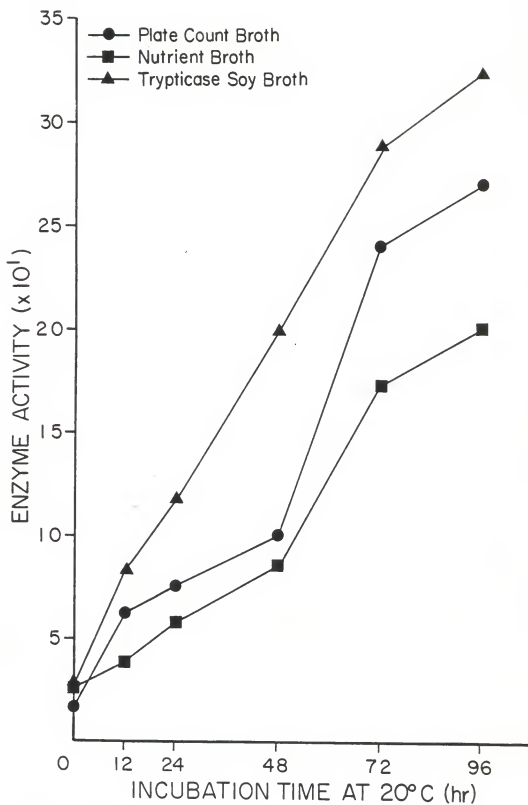
Figure 13. Enzyme activity at 35 C for 15 min (pH 8) of the cell-free broth of <u>Planococcus citreus</u> cells grown in Plate Count Broth, Nutrient Broth and Trypticase Soy Broth for up to 96 hrs.

produced (as measured by enzyme activity) is more clearly observed. After 48 hrs of incubation, the enzyme activity for the P. citreus grown in TSB, PCB and NB was 200, 100 and 85.5 units of activity, respectively. Under the conditions tested, P. citreus exhibited maximum enzyme production when grown in TSB.

The units of activity per cell per hr (m) was calculated for each medium used and the results are shown in Table 6. The average m value for TSB, PCB and NB was 168.50, 105.67 and 59.32, respectively. These data show that the amount of enzyme produced by actively growing P. citreus cells (midlog phase) in TSB is higher than that produced when P. citreus is grown in PCB or NB. The analysis of the data supports this observation. A significant difference ($\alpha = 0.05$ level) was observed between the m values of TSB, PCB and NB (Table 6).

Thus, after evaluating the results from this section, TSB was chosen as the best medium for P. citreus growth and enzyme production and was used for the remainder of the study. The combination of nutrients in TSB allowed for the rapid growth of P. citreus and by doing so, permitted the production of more extracellular proteolytic enzyme. In addition to the combination of nutrients in TSB, the presence of 0.25% dextrose may play a role in extracellular enzyme production. Dextrose has been suggested as a possible inducer of a variety of enzymes (57), although this effect was not specifically tested in these experiments.

Effect of Incubation Temperature on Enzyme Production and Activity

The ability of P. citreus to produce an active extracellular enzyme at 5, 20 and 35 C was investigated in order to determine the ability of the extracellular enzyme(s) to affect shrimp protein at refrigeration (5 C) or iced temperatures. Figure 14 shows the increase in optical

Table 6. Units of enzyme activity per cell per hour (m) of <u>Planococcus</u> <u>citreus</u> grown in Trypticase Soy Broth (TSB), Plate Count Broth (PCB) and Nutrient Broth (NB) at midlog phase.[1]

Medium	mean m value[2]
TSB	148.50[a]
PCB	105.67[b]
NB	59.32[c]

[1]Cells were grown at 20 C and enzyme activity was measured at 35 C for 15 min (pH 8).
[2]average of 6 observations
Means followed by the same letter do not differ significantly at the $\alpha = 0.05$ (r^2 from Anova table 0.984)
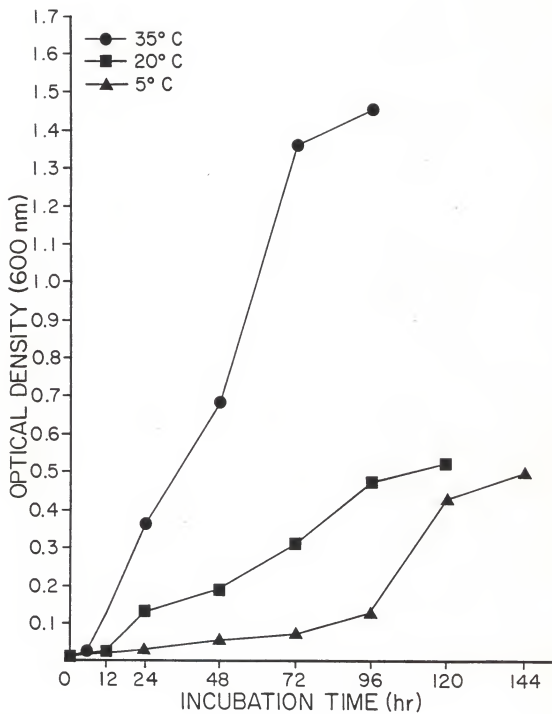
Figure 14. Spectrophotometric growth curves of <u>Planococcus</u> <u>citreus</u> in Trypticase Soy Broth incubated at 5, 20 and 35 C.

density of P. citreus cultures grown at 5 C for 144 hrs, 20 C for 120 hrs and 35 C for 96 hrs. After different time intervals for growth adaptation, P. citreus grew at all three temperatures. Direct microscopic observation of P. citreus cells growing at the three temperatures revealed a difference in cell arrangements. When cells were grown at 35 C, the predominant morphology present was clusters of gram-positive cocci. At this temperature, the cells are rapidly growing and dividing, thus, possibly accounting for the observed predominance of clusters. At 20 C a mixture of tetrads, pairs and single cells were observed which is the predominant morphology exhibited by this organism, as described in Bergey's Manual of Determinative Bacteriology (29). At 5 C the predominant cell arrangement observed was single cells. This particular morphological structure might predominate because of the slow metabolic rate at this temperature, although these rates were not investigated. Individual cells have more surface area for the uptake of nutrients. At 5 C, the movement of nutrients within the cell is slow. Consequently, the increase in surface area is particularly desirable from a nutritional standpoint.

Table 7 illustrates the enzyme activity of the cell-free broth of P. citreus grown at 5 C for 108 hrs, 20 C for 72 hrs and 35 C for 36 hrs (midlog at each temperature) and incubated with shrimp protein at 5, 20 and 35 C for 60, 30 and 15 min, respectively. P. citreus produced an active extracellular enzyme(s) when grown at all temperatures. In addition, the cell-free broth obtained from the three temperatures of growth exhibited activity at all three enzyme-substrate incubation temperatures (5, 20 and 35 C). As the temperature of growth increased from 5 to 35 C, the enzyme activity increased at a similar rate at the three enzyme-

Table 7. Enzyme activity measured at 5, 20 and 35 C (pH 8) of the cell-free broths of Planococcus citreus grown in Trypticase Soy Broth (TSB) at 5, 20 and 35 C for 108, 72 and 36 hrs, respectively.

Enzyme-Substrate Incubation Temperature (C)	Temperature of Growth (C)		
	5	20	35
5	24.18 ± 1.11[a]	58.18 ± 2.08	116.17 ± 8.40
20	25.65 ± 1.20	72.30 ± 2.04	139.67 ± 7.88
35	28.56 ± 1.35	98.55 ± 3.10	178.93 ± 7.68

[a] Average of 6 observations ± standard deviation

substrate incubation temperatures. A higher P. citreus count was observed at 35 C and the production of extracellular enzyme(s) was also higher at all three enzyme-substrate incubation temperatures. This indicates that the amount of enzyme produced by P. citreus is related to the amount of growth of the organism in the medium. In addition, as the enzyme-substrate incubation temperature increased from 5 to 35 C, the enzyme activity of the cell-free broths increased. Although enzyme activity is present at the lower temperatures, the data presented indicate that the optimum temperature of the extracellular protease system may be close to 35 C. Consequently, the results indicate that P. citreus can indeed produce an active extracellular enzyme(s) capable of utilizing the protein in shrimp when shrimp is stored at refrigerated or iced temperatures.

The effect of refrigeration temperatures on enzyme activity has been studied (50,51). In most of the research, the majority of the enzymes studied lost activity when incubated at low temperatures. Studies have shown that lactic streptococci characteristically produced less acid after storage at refrigerated temperatures. Such stored cells also show a diminished residual proteinase activity (49,50,51,52,149). The researchers stated that after storage at 3 C, the enzyme showed gross structural alterations with a concomitant loss of activity. Gel filtration and sedimentation velocity data indicate that inactivation of the enzyme was a result of aggregation to higher molecular weight forms (50). However, several investigators (49,52,131) previously suggested that storage inactivation of enzymes may be caused by induced conformational or structural changes. Scutton and Utter (128) and Havir et al. (81) observed that inactivation of various enzymes by low temperature storage

was due to dissociation of the molecules into subunits. The inactivated enzymes could be reactivated by warming to room temperature.

Purification of the Extracellular Enzyme(s)

Planococcus citreus was grown in Trypticase Soy Broth (TSB) at 20 C for 72 hrs. The cell-free broth was used in the isolation of the extracellular enzyme(s) of this organism. The cell-free broth had a total activity of 1.31×10^5 units of activity (total enzyme activity = change in 0.1 fluorescence units of the TCA filtrate per milliliter of enzyme per minute), 44.52 mg/ml of protein and a specific activity of 29.45 units of activity/mg protein (Table 8). The cell-free broth was then fractionated with 0-55%, 55-70% and 70-100% ammonium sulfate $((NH_4)_2SO_4)$.

After overnight dialysis (16 hrs) in phosphate buffer pH 8, the activity of the 0-55%, 55-70% and 70-100% ammonium sulfate precipitates was measured. Table 9 shows the proteolytic activity of the various fractions examined. The specific activity of each fraction was 4.59, 52.39 and 3.99 units of activity/mg of protein for the 0-55%, 55-70% and 70-100% fractions, respectively. Eighty-six percent of the activity was present in the 55-70% fraction. This is compared to 7.5 and 6.5% for the 0-55% and 70-100% fractions, respectively (Table 9). Ammonium sulfate precipitation is a common method used to precipitate proteins for their purification. As the ammonium sulfate concentration is raised from zero, the solubility of a given protein at first usually increases but then the "salting-in" effect comes to an end and as the salt concentration is raised to higher values a "salting-out" effect is observed and the protein becomes progressively less soluble (65). The major portion of the extracellular proteolytic enzyme(s) of _P. citreus_ was salted

Table 8. Purification of an extracellular proteolytic enzyme from Planococcus citreus.

Fraction	Volume	Total Activity[a] (units x 10³)	Protein[b] (mg/ml)	Specific Activity[c] (units/mg total protein)	Purification	Recovery (%)
Cell-free broth	900	113.3	44.52	29.45		100.0
70% $(NH_4)_2SO_4$ precipitation	350	102.7	19.60	52.39	1.78x	78
Sephacryl[R] S-200 Superfine	115	65.3	1.42	461.65	15.67x	50
DEAE-Sephadex[R] A-50	90	64.0	0.32	780.37	26.50x	49

[a] Determined using gelatin as substrate

[b] Average of duplicate samples

[c] Total activity/Total protein = specific activity (units of activity/mg protein)

Table 9. Proteolytic activity at 35 C for 15 min (pH 8) of various ammonium sulfate fractions of the cell-free broth of *Planococcus citreus*.

Fraction	Total Activity[a] (units x 10⁻³)	Protein[b] (mg/ml)	Specific Activity[c] (units/mg total protein)	Distribution of Activity (%)
0-55%	8.31	18.07	4.59	7.5
55-70%	102.70	19.60	52.39	86.0
70-100%	5.00	12.50	3.99	6.5

[a]Determined using gelatin as substrate
[b]Average of duplicate samples
[c]Total activity/Total protein = specific activity (units of activity/mg protein)

out between 55-70% ammonium sulfate saturation. Table 9 shows that 86% of the activity towards gelatin is observed in this fraction. Table 8 shows that the activity of the 55-70% ammonium sulfate fraction was 1.78 times greater in specific activity than the cell-free broth. A 78% recovery of the extracellular enzyme(s) was achieved in this step of the enzyme purification.

Sephacryl[R] S-200 Superfine, a high resolution chromatographic medium for gel filtration of proteins, nucleic acids, polysaccharides and biopolymers (120), was used to separate the enzyme(s) present in the 55-70% ammonium sulfate fraction according to molecular weight. Figure 15 shows that four protein peaks were recovered after the elution of the enzyme fraction through the Sephacryl[R] S-200 column. However, when the proteolytic activity was measured, the majority of the activity was present in protein peak C (third peak in Figure 15). Peak C had a specific activity of 651.0 units. The enzyme(s) was purified 15.67 times and 50% of the enzyme was recovered in this step (Table 8). The fractions comprising peak C were pooled for further purification. The percent recovery of the extracellular proteolytic enzyme of P. citreus after molecular sieve chromatography using Sephacryl[R] S-200 Superfine was within the range of most of the enzymes recovered when the more traditional Sephadex[R] gels have been used (70,113,114,136).

The pooled fractions of peak C were further rechromatographed using DEAE-Sephadex[R] A-50 (functional group $-C_2H_4N+(C_2H_5)_2H$. A-50 gels are usually used for low and medium molecular weight proteins (up to 200,000). Ion exchange chromatography may be defined as the reversible exchange of ions in solution with ions electrostatically bound to an insoluble support medium. The ion exchanger is the inert support medium
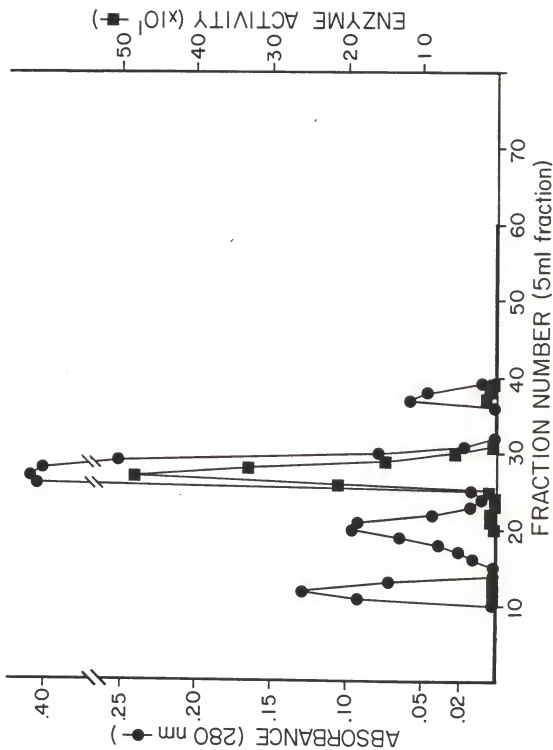
Figure 15. Separation of proteins present in the 55-70 % ammonium sulfate fraction using Sephacryl[R] S-200.

[a] 30 x 2.5 cm column, eluted with 0.02 M phosphate buffer pH 7.

to which is covalently bound positive (in the case of the anionic exchanger) or negative (in the case of a cation exchanger) functional groups (48). A sodium chloride (NaCl) gradient (range of ionic strength, $\mu = 0.11 - 0.23$) was used with the ion exchange column to elute the protein components. A gradient is a physical method of constantly changing the salt concentration of a solution that is being passed through the column creating a constant and linear increase in concentration (48). Figure 16 shows one major peak after ion exchange of the pooled active fractions from peak C. The isolated peak exhibited a specific activity of 780.37 units (Table 8). The proteolytic enzyme was purified 26.50 times and 49% recovery was achieved (Table 8). Fractions 17 to 21 (Figure 16) were pooled for future characterization.

Purity of the Extracellular Proteolytic Enzyme

Many methods can be used to establish the purity of an enzyme preparation. However, the best indication of purity of an enzyme preparation is by the consistent failure to detect heterogeneity when several analytical techniques are used (i.e., a single peak in chromatographic systems, a single band on electrophoresis, a single band after isoelectric focusing and/or one component in solubility or precipitation tests). However, the final criterion for purity is the demonstration of a unique amino acid sequence (61,65) but this is rarely done in order to demonstrate purity.

The recovery of the isolated peak (Figure 16) as a single entity with homogeneous activity after DEAE-Sephadex[R] A-50 ion-exchange chromatography was the first indication that the major extracellular proteolytic activity of P. citreus was isolated in a purified form.
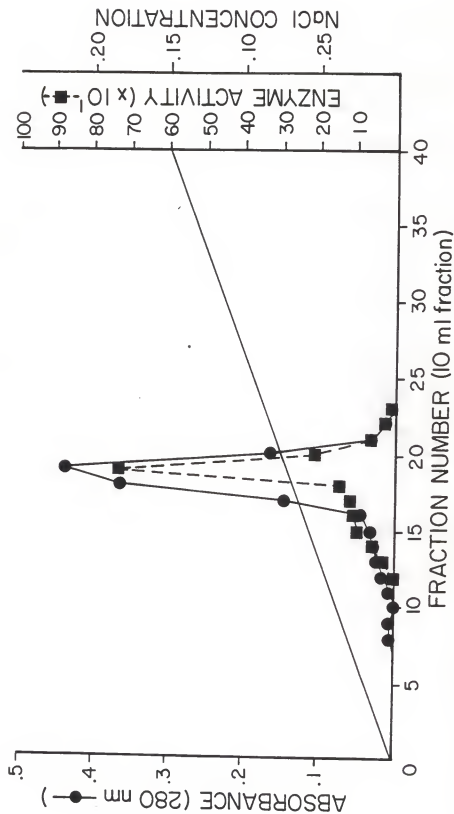
Figure 16. Ion-exchange chromatography using DEAE-Sephadex[R] A-50 of the pooled active fractions obtained in the molecular sieve chromatography step.[a]

[a] 40 x 2.5 column, eluted with 0.02 M phosphate buffer pH 7 with a linear gradient of 0.01 M NaCl- tris 0.15 M NaCl at a rate of 25 ml/hr.

According to Cooper (48) electrophoretic techniques have become principal tools for characterizing macromolecules and for assaying their purity. Figure 17 shows a single band after SDS-PAG electrophoresis using 50 µl of the purified enzyme. A single homogeneous band is indicative of the presence of only one enzyme, i.e., the purity of the extracellular enzyme of P. citreus. In addition, as an additional test for purity, increasing amounts of the purified extracellular enzyme were added to the gels. Enzyme concentrations of 50 µg protein/gel, 100 µg/gel, 150 µg/gel and 200 µg/gel were used. Figure 18 shows that a single band is recovered after SDS-PAG electrophoresis of each protein fraction. Thus, these results add to the evidence indicating the purity of the extracellular proteolytic enzyme of P. citreus. Consequently, an extracellular proteolytic enzyme produced by P. citreus was purified 26.50 times using the procedures outlined previously with 49.0% of the enzyme being recovered (Table 8). The specific activity of the enzyme was 780.37 units of activity/mg protein.

Schwabe (127) reported the use of the fluorescamine reagent to measure proteolytic enzyme activity of cathepsin enzymes using hemoglobin a substrate. He stated that while the fluorescamine reagent has been used successfully for quantitative amino acid analysis, protein and peptide determination, it has also beneficial applications in enzymology. In addition, Schwabe (127) compared the fluorometric technique with the Lowry method (98). He concluded that the fluorometric method was 100 times more sensitive than the Lowry method, much faster and less complicated. The fluorometric technique proved to be an efficient method for the measurement of proteolytic enzyme activity.

Figure 17. Acrylamide gel electrophoresis of the purified enzyme of
Planococcus citreus.

50 μg/gel   100 μg/gel   150 μg/gel   200 μg/gel

Figure 18. Acrylamide gel electrophoresis of increasing concentrations of the purified extracellular enzyme of _Planococcus citreus_.

With the presence of the relatively new Fluorometric technique, that appears to be more sensitive and reproducible than the traditional methods available for measuring proteolytic enzyme activity, the results of various previous research with extracellular enzymes (36,78,84,88, 136) using the Anson method (9) could have possibly resulted in higher recoveries and higher measurable total enzyme activity. The following investigators are some of those who used the Anson method to study the various enzymes. Tarrant et al. (136) working with Pseudomonas fragi in pig muscle isolated an extracellular proteolytic enzyme with only 18% recovery after partial purification. Husein and McDonald (84) characterized an extracellular proteinase from Micrococcus freudenreichii using casein as substrate with 23% recovery after partial purification. Christison and Martin (36) isolated and preliminarily characterized an extracellular protease of Cytophaga spp. using casein, hemoglobin and azocoll as substrates. After chromatography with DEAE-Cellulose[R] only 26% of the enzyme was recovered. Khan et al. (88), looking at the extracellular proteases of Mucor pusillus, isolated and characterized two fractions. However, after DEAE-Sephadex[R] A-50 only 29.3% of the milk-clotting fraction was recovered and 47.0% of the fraction with protease activity toward hemoglobin was recovered. Gnosspelius (76) purified an extracellular protease from Myxococcus virescens using phosphate precipitation, gel exclusion and ion exchange chromatography. Only 20.1% was recovered after the chromatographic step. In the work reported in this dissertation, following DEAE-Sephadex[R] A-50, 49.0% of the extracellular enzyme of P. citreus was recovered when the Fluorometric method was used to measure proteolytic activity.

Characterization of the Extracellular Proteolytic Enzyme

The fractions collected (17-21) from peak B (Figure 16) were pooled and used for the characterization of the extracellular enzyme of $\underline{P}$. citreus.

Molecular Weight Determination

Two methods were used to determine the molecular weight of the enzyme, column chromatography (Sephacryl[R] S-200 Superfine) and acrylamide gel electrophoresis. Standards ranging from a molecular weight of 10,000 to 200,000 were used to determine the molecular weight of the $\underline{P}$. $\underline{citreus}$ enzyme. Using both techniques, the molecular weight of the extracellular enzyme of this organism was approximately 29,000. Figures 19 and 20 show the molecular weight determination using Sephacryl[R] S-200 and acrylamide gel electrophoresis, respectively. Different standards were used in each case to assure that the molecular weight was estimated correctly.

A search of the literature was done in order to compare the molecular weight of the extracellular proteolytic enzyme of $\underline{P}$. $\underline{citreus}$ with extracellular proteases from other microorganisms. Pacaud and Uriel (113) estimated the molecular weight of a protease from $\underline{Escherichia}$ $\underline{coli}$ using electrophoresis on polyacrylamide gels and sucrose-density gradient centrifugation to be about 43,000. Four years later, Pacaud and Richaud (114) estimated the molecular weight of a second protease of $\underline{E}$. $\underline{coli}$ using gel filtration and SDA-acrylamide gels to be 58,000. Drapeau et al. (59) estimated the molecular weight of an extracellular protease of $\underline{Staphylococcus}$ $\underline{aureus}$ to be approximately 12,000 using sedimentation equilibrium and gel electrophoresis studies. Arvidson et al. (12) reported the molecular weight of an extracellular (alkaline protease) enzyme from $\underline{S}$. $\underline{aureus}$ to be approximately 12,500. Later, he reported
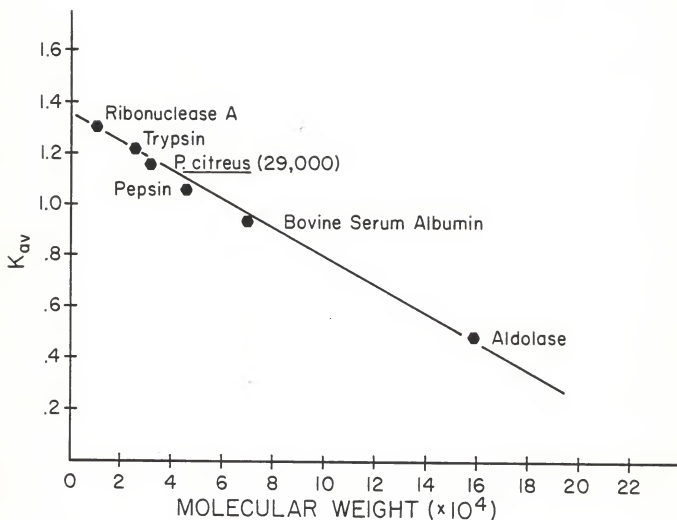
Figure 19. Calibration curve for the molecular weight estimation of
Planococcus citreus proteolytic enzyme using Sephacryl [R]
S-200 column chromatography.[a]

[a]40 x 2.5 column, eluted with 0.02 M phosphate
buffer at a rate of 15 ml/hr.
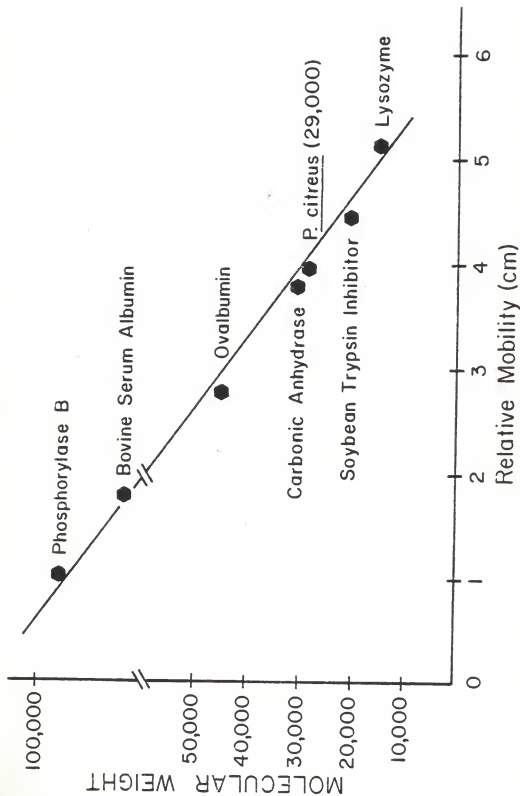
Figure 20. Calibration curve for the molecular weight estimation of *Planococcus citreus* proteolytic enzyme using acrylamide gel electrophoresis.[a]

[a] 10 % acrylamide: Bis 30:0.8 gel, 1–1.5 mAMP/ gel for 3 hrs.

(11) the molecular weight of a EDTA–sensitive S. aureus protease as 28,000. Recently, Hoshida et al. (137) estimated the molecular weight of a proteolytic enzyme from Bacillus sphaericus to be about 26,000. Gnosspelius (76) working with an extracellular enzyme of Myxococcus virescens reported its molecular weight as 26,000. Thus, the apparent molecular weight of the extracellular enzyme of P. citreus (MW 29,000) is within the range of other extracellular proteolytic enzymes reported in the literature.

Effect of Ionic Strength on Enzyme Activity

The effect of salts on the solubility of proteins is well known. The solubility is usually a function of the ionic strength. In conditions of high ionic strength, the ions attract around themselves the polarizable water molecules, making less water available for the proteins since, at high salt concentrations, the number of charged groups contributed by the salts is enormous compared with those of the proteins. Consequently, the solubility of the proteins decreases (152). In addition, any change in the charges of an enzyme may cause various transformations in structure or active site configuration that could affect its activity towards the substrate. Figure 21 shows that ionic strengths (μ) of 0.15–0.83 did not alter the attraction of the P. citreus extracellular enzyme towards gelatin substrate. However, as the ionic strength was increased the activity of the enzyme decreased. An ionic strength of 1.60 (1.5 M NaCl) caused a decrease in enzyme activity of approximately 60%. Thus, if ionic strengths above 0.83 (0.75 M NaCl) are used they may cause a change in solubility of the enzyme, charged groups, conformation of the enzyme, active site stability and/or active site availability to the substrate. Gnosspelius (76) stated that variations in the
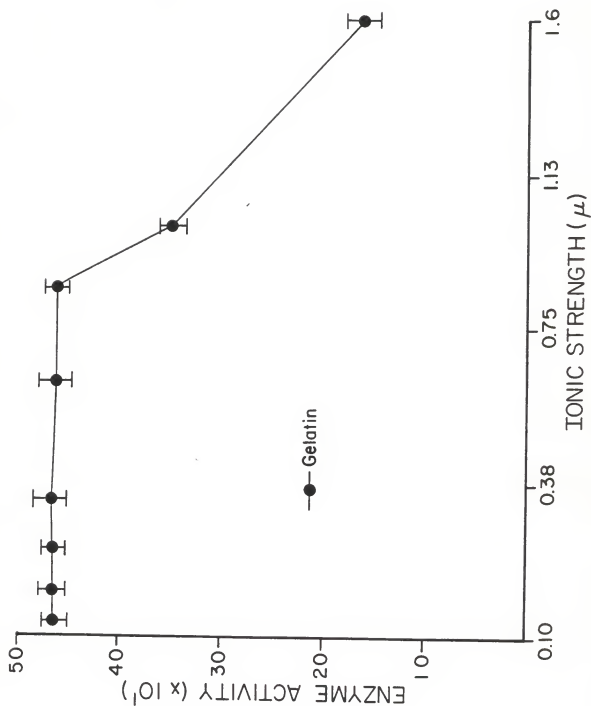
Figure 21. Effect of ionic strenght on the activity of the extracellular proteolytic enzyme of _Planococcus citreus._[a]

[a]Activity assayed at 35 C for 10 min, pH 8.

ionic strength did not signficantly influence the activity of <u>Myxococcus</u> <u>virescens</u> when casein was used as the substrate. However, the actual data for this observation were not presented in the literature.

In that the activity of the proteolytic enzyme was not affected by ionic strengths of $\mu=0.83$ or lower, the buffers shown in page 44, (Determination of Optimum pH) were considered acceptable and were used for the determination of the optimum pH of the <u>P. citreus</u> enzyme.

Optimum pH Determination

The pH optimum of an enzyme is dependent upon a number of experimental parameters. Changes in pH may cause changes in the ionization of prototropic groups (groups capable of ionization) in the active site of an enzyme. These prototropic groups in the active site may be involved in maintaining the proper configuration of the site, in binding a substrate to enzyme and/or in transformation of substrate to products (133). However, there is usually a zone of maximum ion stability in which enzyme activity is maximal. Enzyme inactivation also increases on the acid and alkaline sides of this maximum activity zone. Observing Figure 22, enzyme activity was maximum at pH 8 when both gelatin and shrimp protein were used as substrates. The activity dropped as the pH became more acidic or alkaline. Although not statistically significant, a slightly higher activity was evident at the alkaline pH's (9 and 10) when shrimp protein was used as substrate.

The majority of the bacterial enzymes studied have shown maximum proteolytic activity at neutral pH's (57,65,151). The enzyme isolated in this study resembles the bacterial proteolytic enzyme from <u>Proteus</u> <u>vulgaris</u> (105), <u>Bacillus</u> <u>sphaericus</u> (155), <u>Staphylococcus</u> <u>aureus</u> (11,12), <u>Serratia</u> <u>marcescens</u> (106) and <u>Pseudomonas</u> spp. (87) in that they all
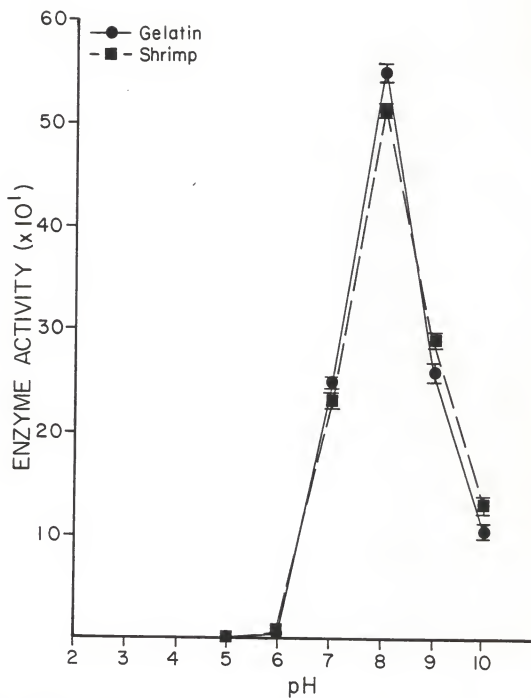
Figure 22. Optimum pH of the extracellular proteolytic enzyme of
<u>Planococcus citreus</u>.[a]

      [a]
      Gelatin and shrimp protein substrate incubated
      at 35 C for 10 min, pH 8.

require a slightly alkaline pH for optimum activity. Considerable activity is present at neutral pH's; the pH of freshly caught shrimp is around neutrality. During shrimp storage, the pH of shrimp will increase (5).

Optimum Temperature Determination

Changes in temperature may affect enzymatic reactions in a number of ways. Some of these effects may include: a) stability of the enzyme; b) affinity of enzymes for activators and inhibitors; c) ionization of prototropic groups; d) enzyme-substrate affinity; and e) velocity of breakdown of enzyme-substrate complex (131). The optimum temperature of the P. citreus extracellular enzyme when both shrimp protein and gelatin were used as substrates was 35 C (Figure 23). Although not statistically signficant, a slightly higher enzyme activity was observed at the lower temperatures (5 and 10 C) using shrimp protein as substrate as compared to gelatin. However, at the higher temperatures (45 and 55 C) the reverse was evident. Thus, as previously observed with the cell-free broth (enzyme crude extract), the purified enzyme of P. citreus can exhibit activity at the temperatures of refrigerated shrimp.

Thermal Stability

The cell-free broths obtained from P. citreus cells grown in Trypticase Soy Broth (TSB) at 5 and 35 C for 108 and 36 hrs, respectively, and the P. citreus purified enzyme were incubated at 35, 45, 55, 65, 75 and 85 C for 15 min in order to study the various temperatures effects on stability. The enzyme activity of the cell-free broths and purified enzyme remaining after the various heat treatments was assayed using gelatin as the substrate and compared to the activity observed with the unheated cell-free broths and purified enzyme. Figure 24 shows the
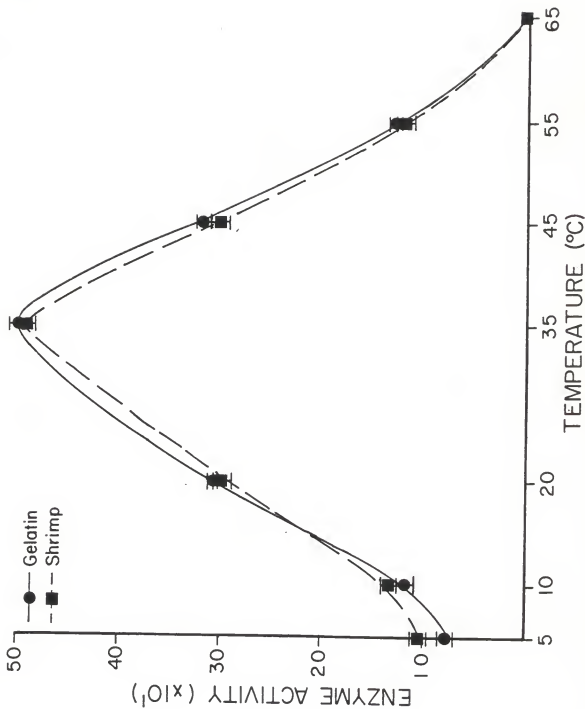
Figure 23. Temperature optimum of the extracellular proteolytic enzyme of _Planococcus citreus_.[a]

[a] Activity assayed at 35 C for 10 min, pH 8.
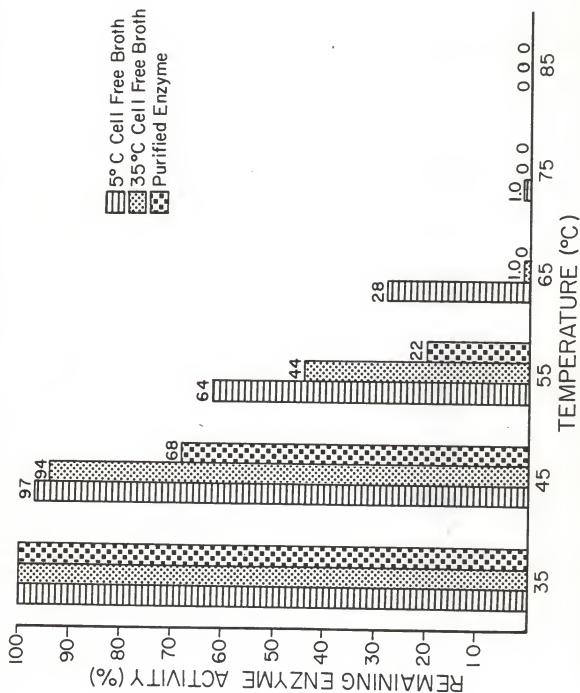
Figure 24. Thermal stability of the enzymes in the cell-free broths of _Planococcus citreus_ grown at 5 and 35 C and of the purified enzyme.[a]

[a] Cell-free broths incubated for 15 min at each temperature and activity assayed at 35 C for 15 min (pH 8) and purified enzyme incubated for 10 min and activity assayed at 35 C for 10 min (pH 8).

thermal stability of the cell-free broth of P. citreus cells grown at 5 and 35 C and of the purified enzyme. In general, as the temperature increased, the activity of all three fractions decreased. After 15 min of incubation at 65 C, all the activity was lost in the purified enzyme fraction and only 1% was left in the 35 C cell-free broth fraction. However, 28% of the activity still remained in the 5 C cell-free broth fraction. After 15 min at 75 C, 1% of the activity of the 5 C cell-free broth fraction still remained. Perhaps the enzymes in the cell-free broth of P. citreus grown and stored at 5 C have undergone a structural change (50,51) or have a slightly different structure than the enzyme produced at 35 C. This change could result in an enzyme conformation with an active site that is more protected from increased temperatures.

Usually, an enzyme is more stable to temperature changes in an intact tissue or in an homogenate where its structure is protected by the presence of other colloidal material (i.e. proteins, carbohydrates, etc.) than it is in a purified form (147,151). However, in general, those enzymes which have molecular weights ranging from 12,000 to 50,000 are composed of single polypeptide chains and having disulfide bonds are usually more resistant to heat treatment. The larger the enzyme and the more complex its structure the more susceptible it is to increases in temperature (151). Figure 24 shows that the cell-free broths are more stable to heat than the purified enzyme.

Effect of Sodium Chloride Concentration

The effect of increasing sodium chloride (NaCl) concentration on enzyme activity was examined. Concentrations of 0–1.50% NaCl were investigated. When shrimp protein was used as the substrate, the activity of the extracellular enzyme increased until 0.50 g/100 ml NaCl (0.5%)

was reached, then the activity started decreasing. However, when gelatin was used as the substrate, salt concentration (0-1.50%) had no apparent effect on the activity of the enzyme (Figure 25). The concentrations of the NaCl solutions used in this study, 0-0.26 M NaCl, have an ionic strength of μ=0.15-0.34 and are not within the ionic strength range that resulted in decreased enzyme activity (Figure 21).

The effect observed when shrimp protein is used as substrate is probably due to an initial increase in solubility of the substrate due to the increase in salt concentration. Structurally, gelatin is a small protein when compared to shrimp protein. Perhaps the increase in solubility allowed an easier enzyme-substrate interaction, thus, accounting for the initial increase in enzyme activity.

The effect of higher concentrations of NaCl on enzyme activity was not investigated. However, Figure 21 illustrates that an ionic strength of 0.83 (0.75 M NaCl) or above resulted in decreased enzyme activity. Reversible inactivation of the enzyme and substrate effects due to higher NaCl concentrations were not investigated.

Arvidson and coworkers (11,12) showed that the activity of both extracellular proteases I and II from Staphylococcus aureus (neutral and alkaline protease, respectively) was reduced by concentrations of 0.5 M NaCl or above. Gnosspelius (76) stated that an NaCl concentration of 0.2 M in the assay mixture had no effect on the activity of the Myxococcus virescens extracellular enzyme. However, higher NaCl concentrations decreased the proteolytic activity.

During the storage of Penaeus shrimp on ice, the salt concentration will decrease due to the leaching of the salt as the ice melts or percolates through the shrimp. As the NaCl concentration decreases in shrimp, the activity of the P. citreus enzyme will be enhanced.
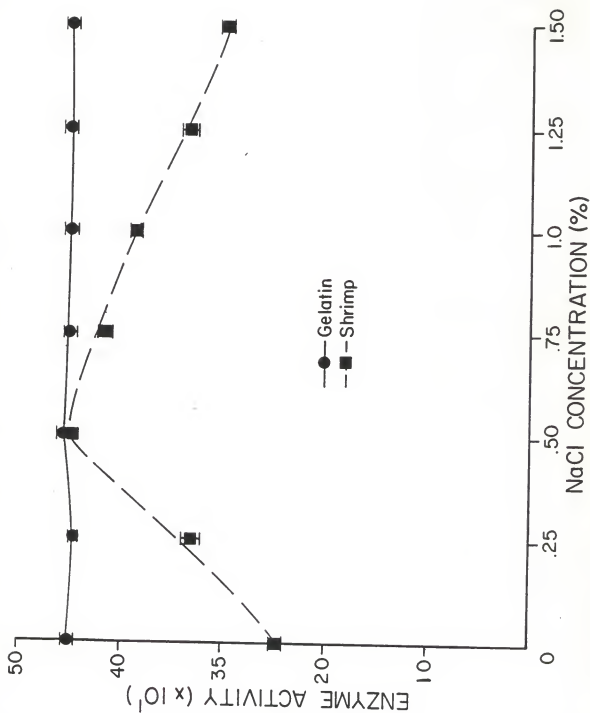
Figure 25. Effect of sodium chloride (NaCl) concentration on enzyme activity.[a]

[a] Activity assayed at 35 C for 10 min (pH 8).

Effects of Sodium Bisulfite Concentration

To control black spotting in shrimp (148), sodium bisulfite ($NaHSO_3$) is used to inhibit enzymatic oxidation of both tyrosine and dihydroxy-phenylalanine thereby preventing darkening of the shell (66,96). Since 1956, agencies such as the former Florida State Department of Conservation (30), now the Department of Natural Resources, have recommended dipping shrimp in a 1.25% sodium bisulfite solution for 1 min to control black spot development. Therefore, the effect of sodium bisulfite concentration (0 to 3%) on the activity of the extracellular proteolytic enzyme of P. citreus was investigated. Figure 26 shows that as the concentration of sodium bisulfite increases, the activity of the enzyme decreases. When sodium bisulfite dissociates in water, it may affect enzyme activity by reducing disulfite (-S=S-) linkages (57). The activity of the enzyme in the presence of 1.25% sodium bisulfite was approximately 240 units of activity. Thus, with the addition of 1.25% sodium bisulfite, approximately 47% of the activity of the proteolytic enzyme was lost. However, as the concentration of sodium bisulfite decreases (leaches out in the melt water) (148) the activity of the enzyme should be less affected.

Effect of Enzyme Concentration

Rates of enzyme-catalyzed reactions are directly dependent on enzyme concentration (151). The effect of enzyme concentration (0 to 200 μl) on the activity of the P. citreus enzyme when gelatin and shrimp protein were used as substrates was investigated. By observing Figure 27, the enzyme preparations used for characterization followed a linear relationship with increasing levels of enzyme. According to Dixon and Webb (57) when the plot passes through the origin, inhibitors are usually
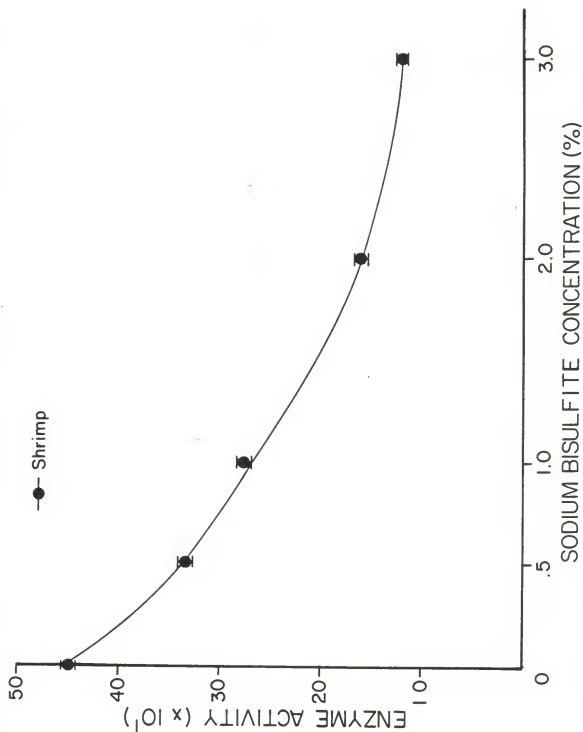
Figure 26. Effect of sodium bisulfite ($NaHSO_3$) concentration on enzyme activity.[a]

[a] Activity assayed at 35 C for 10 min (pH 8).
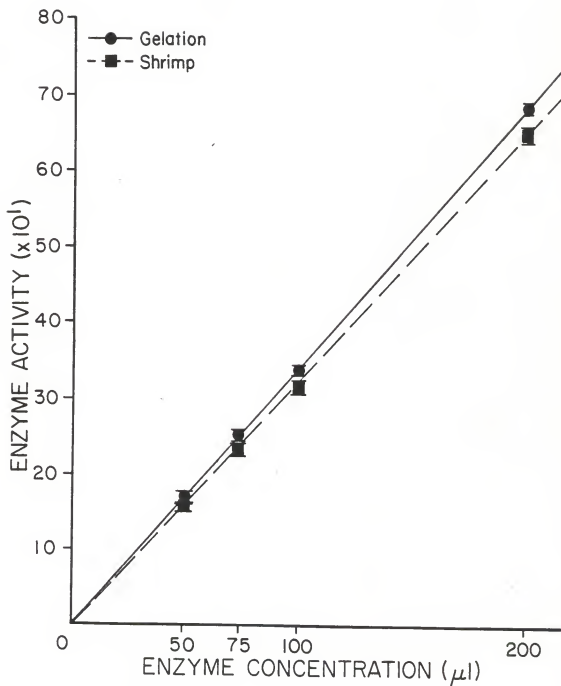
Figure 27. Effect of enzyme concentration on enzyme activity.[a]

[a]Increasing enzyme concentrations were incubated with the substrates at 35 C for 10 min (pH 8).

absent from the preparation. Thus, looking at Figure 27, we can observe

that inhibitors were not present in the preparation.

Effect of Substrate Concentration

Substrate concentration is one of the most important factors which

determine the velocity of enzyme reactions. Figures 28 and 30 illustrate

the effect of substrate concentration on the velocity of the reaction

when gelatin (0 to 1.2 mg/ml) and shrimp protein (0 to 0.6 mg/ml) sub-

strates, respectively, were used. Both enzyme-substrate reactions fol-

lowed Michaelis-Menten kinetics. That is, the enzyme E first combines

with the substrate S to form the enzyme substrate complex ES; the latter

then breaks down in a second step to form the free enzyme E and the

product P: $E + S \xrightarrow[K_{-1}]{K_1} E + P$. Figures 28 and 30 follow the traditional

Michaelis-Menten shape curve (95). Enzyme kinetic calculations (95,152)

were done in order to add to the information about the P. citreus extra-

cellular enzyme and to determine substrate saturation conditions.

The K_m values (Michaelis-Menten constant) calculated in this study

are apparent K_m's. Whole protein substrates (either gelatin or shrimp

protein) rather than specific synthetic amide substrates were used.

True K_m values are calculated using specific substrates and having a

definite knowledge of the enzyme's active site (57). Additional

research is required to demonstrate the active site of the P. citreus

extracellular enzyme.

Apparent K_m and V_{max} values were calculated by transforming the

data in Figures 28 and 30. Double-reciprocal plots (Lineweaver-Burk

plots) were done and they are shown in Figures 29 and 31 for gelatin

and shrimp protein substrates, respectively. The apparent K_m values

for the gelatin and shrimp protein substrates were 0.98 mg/ml and 0.33
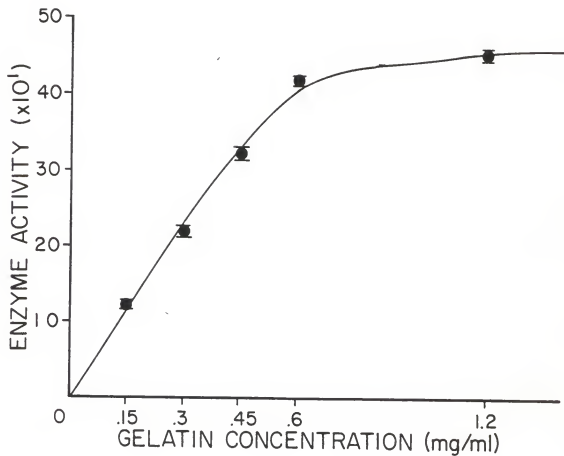
Figure 28. Effect of gelatin concentration on the reaction rate of the
Planococcus citreus extracellular enzyme.[a]

[a]Increasing gelatin concentrations were incubated with
the enzyme at 35 C for 10 min (pH 8).
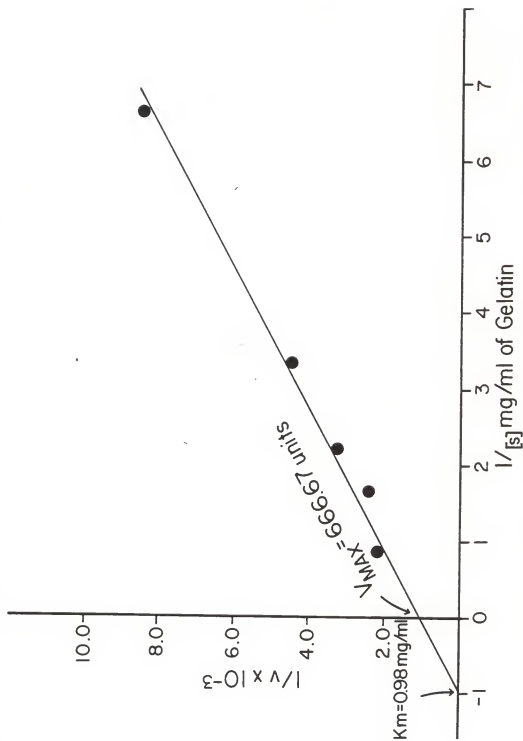
Figure 29. Lineweaver-Burk plot of the Planococcus citreus extracellular enzyme illustrating V_{max} and K_m values using gelatin as the substrate.[a]

[a] Transformation of data presented in Figure 29.
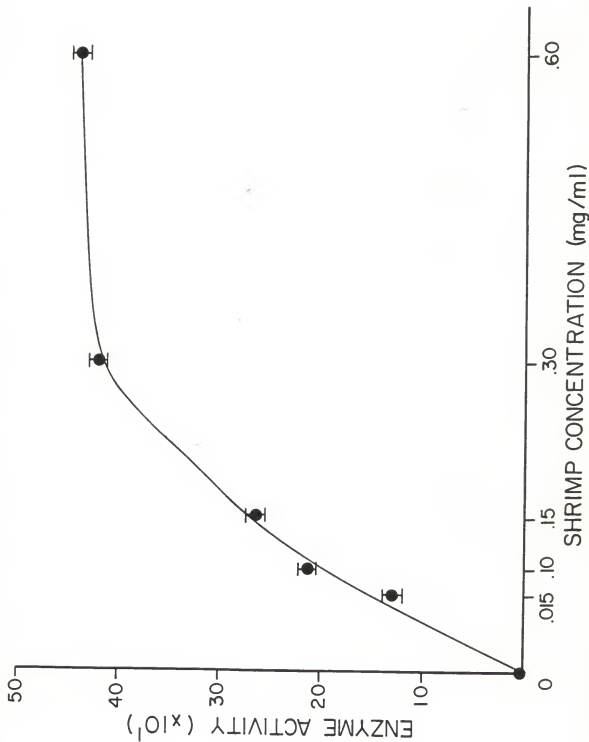
Figure 30. Effect of shrimp protein concentration on the reaction rate of the _Planococcus citreus_ extracellular enzyme.

[a]Increasing shrimp protein concentrations were incubated with the enzyme at 35 C for 10 min (pH 8).
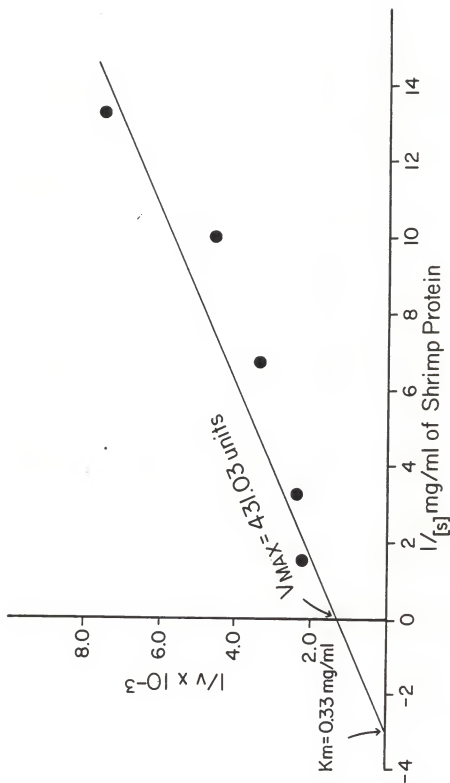
Figure 31. Lineweaver-Burk plot of the *Planococcus citreus* extracellular enzyme illustrating V_{max} and K_m values using shrimp protein as substrate.[a]

[a] Transformation of the data presented in Figure 30.

mg/ml, respectively. The apparent V_{max} values for gelatin and shrimp protein were 666.67 and 431.03 units of activity, respectively. Since K_m is defined as the rate of the disappearance of the enzyme-substrate (ES) complex to the appearance of the ES complex, it appears that gelatin has a higher affinity for the enzyme than shrimp protein. Consequently, V_{max} is lower with shrimp protein as substrate. This is shown in Figures 29 and 31. $\underline{P}$. $\underline{citreus}$ extracellular proteolytic enzyme can utilize and degrade gelatin at a more rapid rate than shrimp protein. Various factors could be responsible for observing a higher V_{max} when gelatin is used as substrate. Gelatin, with a molecular weight of 90,000 (152), is a simple protein when compared to shrimp protein. Four amino acids comprise 70% of the total amino acid composition. Glycine and alanine add to approximately 50% of the amino acids present in gelatin (152). Possibly, the relative simplicity of gelatin makes this protein more available to the action of the $\underline{P}$. $\underline{citreus}$ extracellular enzyme. In addition, gelatin showed higher solubility than shrimp protein in the buffer system used. Perhaps, this increased solubility allowed for an easier enzyme-substrate interaction. However, shrimp protein has a more complex primary structure, and as it was prepared in this study, it is probably a mixture of proteins. Eighteen amino acids comprise approximately 60% of the total amino acid composition (53). The composition of the shrimp protein preparation probably makes it a more complex substrate for the $\underline{P}$. $\underline{citreus}$ extracellular enzyme. Consequently, shrimp protein is not as easily available for the reaction with the enzyme.

Effect of Metal Ions on Enzyme Activity

The effect of various metal ions on the activity of the extracellular proteolytic enzyme of $\underline{P}$. $\underline{citreus}$ was investigated. The effect of

two concentrations of each metal ion is shown in Table 10. Calcium chloride ($CaCl_2$) increased the activity of the proteolytic enzyme, while ferric chloride ($FeCl_3$), mercuric chloride ($HgCl_2$) and potassium chloride (KCl) all suppressed the activity of the enzyme to some extent. Zinc chloride ($ZnCl_2$), magnesium chloride ($MgCl_2$) and manganese chloride ($MnCl_2$) had little effect on enzyme activity.

Divalent cations, except $CaCl_2$, had no appreciable effect on the activity of the extracellular enzyme. The ionic radius of divalent cations is such that one of their primary functions is to coordinate the substrate to the active site (37). Mg and Ca, the alkaline earth cations, participate in the formation of the catalytically active conformation.

Monovalent ions are usually involved in transport mechanisms in the cell (37), thus, usually not as an integral part of the active center. Possibly, K^+ ions could be displacing a required ion resulting in the suppression of enzyme activity. In contrast, Figure 25 showed that NaCl concentrations had no effect on enzyme activity when gelatin was used as the substrate. This apparent effect of K^+ but not of Na^+, possibly related to the larger ionic radius of K^+ ions, should be the subject of additional investigations.

Salts of heavy metals, such as silver, copper, mercury and lead, react rapidly and at low concentrations with sulfhydryl groups; however, they also react with other groups including the imidazole, carboxyl and peptide groups (151). At high concentrations, heavy metals can inhibit by neutralizing charges on the protein and/or by forming cross linkages between protein molecules (57). Iron salts have been found to activate some enzymes but suppress others (57). Some metal ions can activate enzymes by: 1) becoming an integral part of the active site; 2) linking

Table 10. Effect of various metal ions on the activity (assayed at 35 C for 10 min (pH 8)) of the _Planococcus citreus_ extracellular enzyme.

Metal Ion	Concentration (mM)	Enzyme Activity[a]	% Residual Activity[b]
none (control)		458.37 ± 2.08	100.0
$CaCl_2$	10	492.89 ± 1.57	107.5
	20	553.61 ± 2.10	120.7
$FeCl_3$	1	153.61 ± 2.97	33.5
	20	105.33 ± 2.87	23.0
$MgCl_2$	10	460.16 ± 2.77	100.4
	20	470.11 ± 2.85	102.6
$HgCl_2$	1	285.71 ± 2.41	62.3
	20	243.11 ± 1.44	53.0
$MnCl_2$	10	430.17 ± 0.75	93.8
	20	408.16 ± 1.89	89.1
$ZnCl_2$	10	449.95 ± 2.24	98.2
	20	435.00 ± 1.89	94.9
KCl	5	287.72 ± 2.10	62.8
	20	247.67 ± 6.37	54.0

[a] Average of 6 observations
[b] Compared to the control sample

the enzyme with the substrate; 3) changing the equilibrium constant of enzyme reaction; 4) changing surface charge of enzyme; 5) removing inhibitors; and 6) inducing a more active enzyme conformation (151).

Kato et al. (87) reported that calcium chloride ($CaCl_2$) and magnesium chloride ($MgCl_2$) activated the enzyme of a marine-psychrotrophic bacterium (Pseudomonas spp.) and mercuric chloride ($HgCl_2$) and ferric sulfate ($FeSO_4$) suppressed the enzyme. Arvidson (11) reported that magnesium chloride ($MgCl_2$), zinc chloride ($ZnCl_2$) and calcium chloride ($CaCl_2$) activated a protease from Staphylococcus aureus. However, Pacaud and Uriel (112) stated that calcium chloride ($CaCl_2$), manganese chloride ($MnCl_2$) and ferric chloride ($FeCl_2$) activated an enzyme from Escherichia coli but magnesium chloride ($MgCl_2$), zinc chloride ($ZnCl_2$) and mercuric chloride ($HgCl_2$) had no effect on the activity. The results of this study indicate the diversity of effects ions can have on the activity of the enzyme produced by P. citreus.

Effect of Various Reagents on Enzyme Activity

Various reagents were tested to observe their effect on the activity of the extracellular proteolytic enzyme of P. citreus. None of the reagents tested activated the enzyme (Table 11). Dioxane, one of the reagents used in the enzyme activity assay, had no apparent effect on the activity of the enzyme at either concentration examined (10 and 20 mM). The percent residual activity observed was 98.9 and 98.6%, respectively. The results observed with trichloroacetic acid (TCA, 5 and 10%) indicate that both concentrations can terminate the activity of the enzyme (2.0 and 0.0% residual activity, respectively). These results are comparable to those observed in Figure 6.

Table 11. Effect of various reagents on the enzyme activity (assayed at 35 C for 10 min (pH 8)) of the Planococcus citreus extracellular enzyme.

Reagent	Concentration	Enzyme Activity[a]	% Residual Activity[b]
None (control)		449.95 ± 5.59	100.00
EDTA	10 mM	87.72 ± 2.64	19.50
	20 mM	58.50 ± 2.33	13.00
Citric Acid	10 mM	150.72 ± 3.85	33.50
	20 mM	129.72 ± 6.30	28.70
Formaldehyde	1 mM	286.33 ± 2.59	63.60
	20 mM	239.56 ± 2.83	53.20
KCN	1 mM	389.89 ± 2.86	86.70
	20 mM	363.45 ± 3.50	80.80
KMnO$_4$	1 mM	9.28 ± 1.56	2.10
	20 mM	0.00 ± 0.00	0.00
TCA	5%	8.87 ± 1.75	2.00
	10%	3.05 ± 0.93	0.68
Cysteine	1 mM	209.83 ± 4.21	46.60
	20 mM	181.95 ± 6.50	40.40
p-mercaptoethanol	1 mM	284.17 ± 6.23	63.20
	20 mM	247.00 ± 3.28	54.90
p-Dioxane	10 mM	445.17 ± 3.40	98.90
	20 mM	443.45 ± 3.00	98.60

[a] Average of 6 observations ± standard deviation
[b] Compared to the control sample

The enzyme was inactivated by citric acid and EDTA, which act as metal ion chelators. Considerable activity was lost by the enzyme when cysteine and p-mercaptoethanol were added. These reagents reduce and interchange with sulfhydryl groups (SH groups) in proteins resulting in a possible reorganization of the enzyme structure (98). In addition, cysteine can also bind and remove metals (57,155). Possibly, these two compounds might be binding a required trace metal resulting in the observed suppression of enzyme activity. The enzyme lost practically all the activity when 1 mM potassium permanganate ($KMnO_4$) was added (2.10% residual activity) and all the activity when 20 mM $KMnO_4$ was added. When 1 mM or 20 mM of formaldehyde was added, 63.6 and 53.2% residual activity, respectively, was observed. These results are comparable to those observed when p-mercaptoethanol was added. Formaldehyde can react with SH groups forming methylene bridges between amino acids and amide groups. However, it can also link α-amino acids (73), possibly making them unavailable for the reaction with the Fluoram[R] reagent. Potassium cyanide (KCN) affected the activity of the enzyme to a small degree. Cyanide groups are known to combine with cofactors (metal ions) in substrates of enzymes when a C=O group is involved (37). Thus, CN is usually a carbonyl group inhibitor.

Numerous researchers (12,36,87,113,119,157) have reported on the effect of various reagents on extracellular proteases. Kato et al. (87) reported that $KMnO_4$, EDTA and citrate inactivated a _Pseudomonas_ spp. protease while KCN and p-Dioxane did not affect the activity of the enzyme. Yoshida et al. (157), Christison and Martin (36) and Arvidson (12) stated that EDTA and citrate inhibited the proteolytic enzymes from _Bacillus sphaericus_, _Cytophaga_ spp. and _Staphylococcus aureus_. However,

a protease from <u>Escherichia coli</u> (113) was not inhibited by EDTA, cysteine nor p-mercaptoethanol.

The results of this study indicate that the <u>P. citreus</u> enzyme may contain a metal cofactor and possibly sulfhydryl groups. Additional work should be conducted to confirm these findings.

Dipeptidase Activity

Table 12 shows the activity of the purified <u>P. citreus</u> extracellular enzyme towards five synthetic dipeptides. Although lower when compared to the activity on the whole protein substrates, enzyme activity was observed in all five peptides. The highest activity was observed with DL-alanylglycine (47.67 units of activity). In order to make predictions on the specificity of this enzyme, additional peptides should be investigated. Consequently, the extracellular enzyme of <u>P. citreus</u> can utilize and degrade dipeptides to their constituent amino acids.

Enzyme Classification

According to the International Union of Biochemistry scheme (54) for numbering enzymes, the <u>P. citreus</u> proteolytic enzyme would be class-ified as: 3.4.1 (acting on peptide bonds, an α-amino-acyl-peptide hydrolase). The data presented in this study point to the possibility of having an aminopeptidase enzyme; however, further studies with synthetic peptides are necessary for the complete classification of the <u>P. citreus</u> enzyme. In addition, studies need to be conducted to deter-mine if the <u>P. citreus</u> enzyme exhibits endo or exopeptidase activity.

Enzyme Induction Studies

Various media were used in order to determine if the extracellular proteolytic enzyme produced by <u>P. citreus</u> was induced by shrimp protein. Induction is the complete de novo synthesis of enzyme molecules in the

Table 12. Dipeptidase activity of the Planococcus citreus extracellular
           enzyme.

Dipeptide	Enzyme Activity[a]
DL-leucylglycine	11.67 ± 0.89
DL-leucyl-DL-alanine	22.94 ± 0.44
L-leucyl-L-Tryptophane	32.61 ± 1.98
glycyl-DL-leucine	11.56 ± 0.18
DL-alanylglycine	47.67 ± 2.48

[a]Average of 6 observations ± standard deviation

presence of a specific substrate (28,57,73). A substantial number of bacterial exo-enzymes appear to be induced by their substrate or closely related compounds (122) while others are continuously being synthesized by the microorganisms during their growth. According to Pollock (121), enzyme induction does not introduce a new pattern of protein structure into the cell. Whether constituent or induced, and whatever inducer is used, the enzyme formed appears to be identical (121).

Yeast Carbon Base (YCB), a minimal substrate level medium, was used in this study (see Table 4) and was fortified with shrimp protein and/or yeast extract. In addition, a study with Trypticase Soy Broth (TSB) fortified with shrimp protein was also done.

Figure 32 shows the growth of P. citreus in the various media fol-lowing 96 hrs of incubation at 20 C. Overall, P. citreus grew poorly in all four media tested. About a 1.15 log increase in P. citreus cell number was observed in YCB, a 1.20 log increase in YCB + 1.0% shrimp protein (YCBS), a 1.40 log increase in YCB + 0.1% yeast extract (YCBY) and a 1.60 log increase in YCB + 1.0% shrimp protein + 0.1% yeast extract (YCBSY). Therefore, as the nutrients in the growth medium increased, improved growth of P. citreus was observed.

The proteolytic activity of the cell-free broth of P. citreus grown in Yeast Carbon Base supplemented with shrimp protein and/or yeast extract is shown in Figure 33. After 96 hrs at 20 C, the proteolytic enzyme activity of the cell-free broths from each medium was 141, 114, 90 and 77.5 units for YCBSY, YCBY, YCBS and YCB, respectively. Thus, as observed with the P. citreus growth data (Figure 32), as the nutritional composition of the growth medium increased, the amount of enzyme produced also increased (as measured by an increase in total enzyme activity).
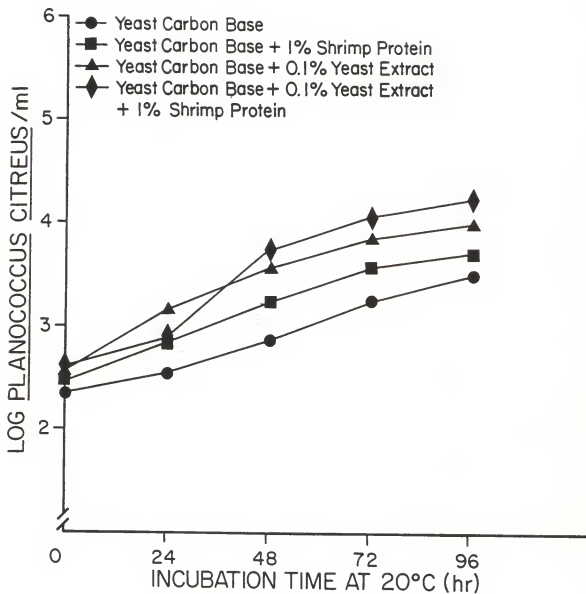
Figure 32. Growth of <u>Planococcus</u> <u>citreus</u> in Yeast Carbon Base
supplemented with shrimp protein and/or yeast extract
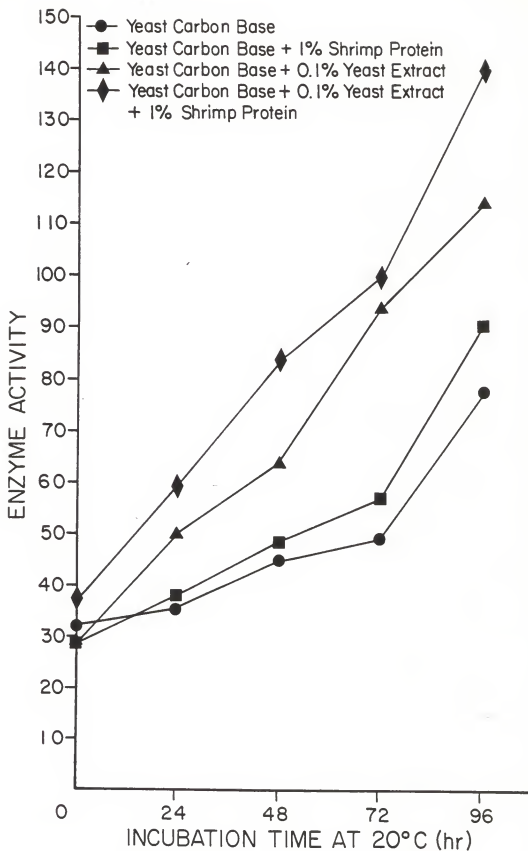at 20 C.

Figure 33. Proteolytic enzyme activity of the cell-free broth of <u>Planococcus</u> <u>citreus</u> grown in Yeast Carbon Base supplemented with shrimp protein and/or yeast extract.

The units of enzyme activity per cell per hr (m) was calculated for each medium used. Table 13 shows that the average m value for YCB, YCBS, YCBY and YCBSY was 4.77, 7.62, 27.82 and 37.12, respectively. These data agree with the results in Figures 32 and 33. As the nutrients in the growth medium increased, more enzyme was produced per P. citreus cell. Thus, it seems that the nutritional composition of the medium of growth, not the mere presence of shrimp protein, influences enzyme production. No significant difference ($\alpha=0.05$ level) was found between YCB (control) and YCB with shrimp protein added (Table 13). The results presented indicate that the enzyme produced by P. citreus is constantly being produced as long as there is cell growth. Consequently, the enzyme is constituent.

The results shown in these induction studies reflect the production of an enzyme by P. citreus in a minimal medium in which the maximum growth attained was 1.6 logs after 96 hrs of incubation at 20 C. Figure 34 shows the growth of P. citreus cells in Trypticase Soy Broth (TSB) with and without shrimp protein added. TSB was previously chosen as the best medium for P. citreus growth and enzyme production (see Growth Medium and Enzyme Production section). P. citreus log count increased approximately 3.75 and 3.80 logs in TSB and TSB + 1.0% shrimp protein, respectively. Thus, P. citreus grew equally as well as in media with or without shrimp protein added.

Figure 35 shows the proteolytic activity of the cell-free broth of P. citreus cells grown in TSB with and without shrimp protein. After 96 hrs at 20 C, the proteolytic enzyme activity of the cell-free broths was 297 and 295 units of activity for TSB and TSB + 1.0% shrimp protein, respectively. Thus, these data support the previous results in showing

Table 13. Units of enzyme activity per cell per hr (m) of <u>Planococcus</u> <u>citreus</u> grown in Yeast Carbon Base supplemented with shrimp protein and/or yeast extract at midlog phase.

Medium	Mean m^1
Yeast Carbon Base (YSB)	4.77^a
Yeast Carbon Base + 1.0% shrimp protein (YCBS)	7.62^a
Yeast Carbon Base + 0.1% yeast extract (YCBY)	27.82^b
Yeast Carbon Base + 1.0% shrimp protein + 0.1% yeast extract (YCBSY)	37.12^c

[1] average of 6 observations

Means followed by the same letter do not differ significantly at the $\alpha=0.05$ level (r^2 from Anova table 0.978)
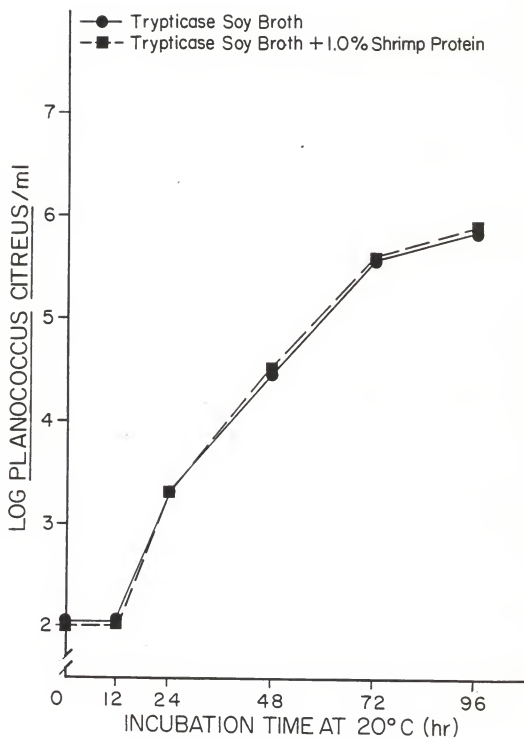
Figure 34. Growth of _Planococcus_ _citreus_ in Trypticase Soy Broth with and without shrimp protein at 20 C.
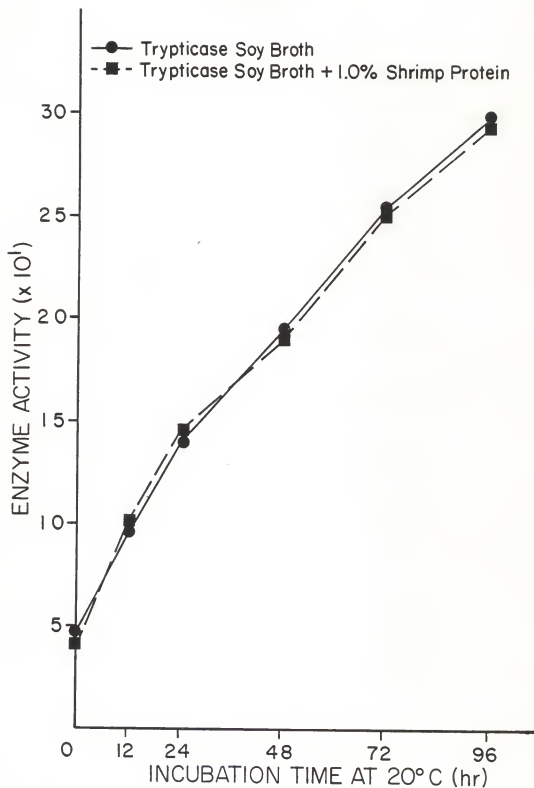
Figure 35. Proteolytic enzyme activity of the cell-free broth of
<u>Planococcus</u> <u>citreus</u> grown in Trypticase Soy Broth with
and without shrimp protein.

that P. citreus can produce an extracellular enzyme(s) in the presence or absence of shrimp protein in the growth medium. The units of enzyme activity per cell per hr (m) of the enzyme produced in TSB and TSB + 1.0% shrimp protein were 147.04 and 146.58, respectively. These values are not significantly different (α=0.05 level). However, when compared to the m values in Table 13, both of these m values are significantly different (α=0.05 level).

Consequently, these data appear to indicate that the extracellular proteolytic enzyme produced by P. citreus is not induced by the presence of shrimp protein in the growth medium. The enzyme is produced in minimal media and its activity per cell per hr increases as the nutrient composition in the medium increases. P. citreus produces the extracellular enzyme even in the presence of surplus nutrients. Table 14 illustrates these findings more clearly. There appears to be no effect, e.g., gene repression, by any of the factors present in the various media used. Consequently, the production of the extracellular proteolytic enzyme by P. citreus appears to be related more to growth of the organism than to the presence of any specific nutrient.

Table 14. Units of enzyme activity per cell per hr (m) of _Planococcus_
citreus grown in various media.

Medium	Mean m value[1]
Trypticase Soy Broth (TSB)	147.68^a
Trypticase Soy Broth + 1.0% shrimp protein	146.58^a
Plate Count Broth (PCB)	105.67^b
Nutrient Broth (NB)	59.32^c
Yeast Carbon Base + 1.0% shrimp protein + 0.1% yeast extract (YCBSY)	37.11^d
Yeast Carbon Base + 0.1% yeast extract (YCBY)	27.82^e
Yeast Carbon Base + 1.0% shrimp protein (YCBS)	7.62^f
Yeast Carbon Base (YCB)	4.77^f

[1] Average of 10 observations for TSB, 4 for TSB + 1.0% shrimp protein
and 6 for the other media used

Means followed by the same letter do not differ significantly at the
$\alpha=0.05$ level (r^2 from Anova table 0.994)

SUMMARY AND CONCLUSIONS

An extracellular proteolytic enzyme from a marine organism, Plano-coccus citreus, was isolated, purified and characterized. Ammonium sulfate precipitation, Sephacryl^R S-200 Superfine chromatography and DEAE-Sephadex^R A-50 ion exchange chromatography were used to purify the enzyme. A single band present after acrylamide gel electrophoresis, as well as chromatography, showed the purity of the extracellular proteolytic enzyme. In addition, the fluorometric technique proved to be an efficient, fast and economical (less enzyme is required) method for the measurement of enzyme activity.

Each fraction of the P. citreus cells studied (extracellular, whole cells, washings of whole cells, soluble intracellular and cellular particulate) exhibited proteolytic activity. However, the major portion, greater than 95.0%, of the active enzyme towards the high molecular weight substrates (gelatin and shrimp protein) was recovered in the extracellular fraction.

Trypticase Soy Broth (TSB), a highly nutritious medium, proved to be the best medium for culturing P. citreus cells and for the production of the extracellular proteolytic enzyme. A highly significant difference ($\alpha=0.05$ level) was observed between the units of enzyme activity produced per cell per hr (m) by P. citreus grown in Trypticase Soy Broth, Plate Count Broth and Nutrient Broth.

The following properties were characteristic of the P. citreus extracellular proteolytic enzyme:

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1) The cell-free broth obtained from P. citreus cells grown at 5 C for 108 hrs, 20 C for 72 hrs and 35 C for 36 hrs exhibited enzyme activity towards shrimp protein at all three enzyme-substrate incubation temperatures (5, 20 and 35 C). Thus, P. citreus when grown at 5 C produces an extracellular enzyme capable of utilizing the protein in shrimp stored either at refrigeration or higher temperatures.

2) The major portion of the extracellular proteolytic enzyme of P. citreus was recovered at an ammonium sulfate concentration between 55-70% saturation. Eighty-six percent of the total activity was recovered in this fraction.

3) Using the fluorometric method for activity measurements, the protease was purified 26.50 fold with a recovery of approximately 49%. The specific activity of the purified enzyme was 780.37 (units of activity/mg of protein).

4) Purity of the enzyme was demonstrated by the presence of a single band after acrylamide gel electrophoresis using various protein concentrations as well as by the presence of a single peak with homogeneous activity after ion-exchange chromatography.

5) The molecular weight of the P. citreus enzyme was approximately 29,000 according to column chromatography using Sephacryl[R] S-200 and acrylamide gel electrophoresis.

6) Ionic strengths of 0.15-0.83 had no effect on the activity of the extracellular enzyme.

7) pH optimum of the proteolytic enzyme was 8. Activity of the enzyme decreased as the pH deviated from this optimum.

8) The optimum temperature for the P. citreus enzyme was 35 C; however, activity was observed at 5 C.

9) After 15 min of incubation of the purified enzyme at 65 C, no activity was observed and only 1.0% activity remained in the cell-free broth of $\underline{P}$. $\underline{citreus}$ grown at 35 C for 36 hrs. However, after 15 min at 75 C, 1.0% activity still remained in the cell-free broth of $\underline{P}$. $\underline{citreus}$ cells grown at 5 C for 108 hrs. Results indicated that the enzyme system in the crude preparations (cell-free broths) was less affected by temperature changes than the purified enzyme. In addition, the enzyme system produced by $\underline{P}$. $\underline{citreus}$ grown at 5 C was more stable to changes in temperature than the 35 C crude enzyme preparation. Perhaps the enzymes present in the 5 C crude extract have an enzyme configuration that better protects the active site from temperature changes.

However, when shrimp is processed (boiled, canned or broiled), the enzyme should be easily inactivated.

10) When shrimp protein was used as substrate, the activity of the enzyme increased as the sodium chloride (NaCl) concentration increased up to 0.5% NaCl. Enzyme activity decreased with higher concentrations of NaCl (0.5–1.5%). When gelatin was used as the substrate, NaCl concentrations (1–1.5%) had no effect on enzyme activity. The increase in NaCl concentration up to 0.5% might have caused an increase in shrimp protein solubility, thus, making shrimp protein more available for the $\underline{P}$. $\underline{citreus}$ enzyme.

11) As the concentration of sodium bisulfite ($NaHSO_3$) was increased from 0 to 3%, the activity of the protease decreased (first-order reaction). Approximately 47% of the activity was lost when 1.25% sodium bisulfite was present in the medium.

12) As the concentration of enzyme increased (0–200 μl), the rate of the reaction increased when gelatin and shrimp protein were used as substrates.

13) Michaelis-Menten kinetics were followed when gelatin and shrimp protein were used a substrates.

14) The apparent K_m values for gelatin and shrimp protein were 0.98 mg/ml and 0.33 mg/ml, respectively. The apparent V_{max} values were 666.67 and 431.03 (units of activity), respectively. This indicates that the P. citreus extracellular enzyme can degrade gelatin faster than shrimp protein.

15) $FeCl_3$, $HgCl_2$ and KCl inhibited the enzyme to some extent, while $CaCl_2$ activated the extracellular enzyme. $ZnCl_2$, $MgCl_2$ and $MnCl_2$ had no appreciable effect on the activity of the proteolytic enzyme. The repressing effect of Fe^{+3} and Hg^{+2} on the activity of this extracellular enzyme may indicate that the enzyme contains sulfhydryl groups. p-Dioxane had no effect on the activity of the proteolytic enzyme. EDTA, citric acid, cysteine, p-mercaptoethanol, potassium permangate and formaldehyde inactivated the enzyme to different degrees indicating that the extracellular enzyme was affected by metal chelators. These results indicate that the enzyme may contain a metal ion as cofactor and possibly sulfhydryl groups.

16) The proteolytic enzyme of P. citreus exhibited activity against the following dipeptides: DL-leucylglycine, DL-leucyl-DL-alanine, L-leucyl-L-tryptophane, glycyl-DL-leucine and DL-alanylglycine. The highest activity was observed with DL-alanylglycine.

17) Preliminary classification of the enzyme shows that it is an α-amino-acyl-peptide hydrolase (3.4.1). Additional studies with synthetic peptides are necessary for complete classification.

18) The extracellular proteolytic enzyme produced by P. citreus was not induced by the presence of shrimp protein in the growth medium.

The enzyme appears to be produced continuously during growth of the organism.

Results obtained under the conditions of these investigations indicate that _Planococcus_ _citreus_ produces an extracellular enzyme which is active at refrigerated temperatures and capable of degrading shrimp protein. In addition, this enzyme is capable of cleaving dipeptides to their constituent amino acids. Consequently, the production of this enzyme by _P._ _citreus_ while growing on shrimp may contribute to the overall decrease in shrimp quality during iced or refrigerated storage.

The function of the extracellular enzyme appears to be one of supplying nitrogenous compounds to the cell. _P._ _citreus_ does not actively utilize carbohydrates for growth, rather its metabolism is directed towards the utilization of proteins. In a nutrient limited marine environment, it appears advantageous from an evolutionary standpoint to produce a single extracellular enzyme of broad specificity. This dependence upon nitrogen compounds by _P._ _citreus_ could be one reason for the unique ecological association that exits between _P._ _citreus_ and shrimp in nature.

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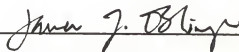
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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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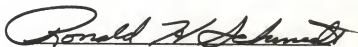
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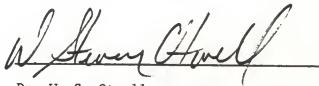
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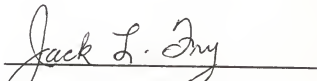Associate Professor of Food Science
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